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<b>(21) International Application Number:</b> PCT/EP97/03358  <b>(22) International Filing Date:</b> 26 June 1997 (26.06.97)  <b>(30) Priority Data:</b> MI96A001309 27 June 1996 (27.06.96) IT  <b>(71) Applicant (for all designated States except US):</b> DOX-AL ITALIA S.P.A. [IT/IT]; Via E. Fermi, 2, I-20050 Correzzana (IT).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> VOLPATO, Ivo [IT/IT]; Via C. Cattaneo, 22, I-06070 S. Mariano (IT). BIZZINI, Bernard [FR/FR]; Le Cerisaie, Roussilhe, F-46100 Figeac (FR). GRABITZ, Ernst, Bernhard [DE/IT]; Via Sant'Eurosia, 22, I-22064 Campofiorengo Fraz. di Casaten- ovo (IT).  <b>(74) Agent:</b> GERVASI, Gemma; Notarbartolo & Gervasi, Corso di Porta Vittoria, 9, I-20122 Milano (IT).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> IgG IMMUNOGLOBULINS AND F(ab') <sub>2</sub> FRAGMENTS THEREOF, SPECIFIC FOR DRUGS AND METABOLITES THEREOF, AND THEIR USE FOR DETOXIFICATION PURPOSES  <b>(57) Abstract</b>  A description is given of F(ab') <sub>2</sub> fragments and corresponding IgG immunoglobulins, active as specific antibodies to drugs and metabolites thereof, and their use in the treatment for detoxifying humans and animals treated with said drugs. Said fragments are obtained by removing the Fc fragment of IgG immunoglobulins obtained from the serum of animals treated with immunogen derivatives of said drugs, which, on their turn, are obtained by conjugating said drugs with proteic or polysaccharide carriers. The description also sets forth a competitive immunodiagnostic method for the determination of drugs in biological fluids and the corresponding diagnostic kit.		

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**IgG IMMUNOGLOBULINS AND F(ab')<sub>2</sub> FRAGMENTS THEREOF, SPECIFIC FOR DRUGS AND METABOLITES THEREOF, AND THEIR USE FOR DETOXIFICATION PURPOSES**

specific antibodies to drugs and metabolites thereof, and their use in the treatment for detoxifying humans and animals treated with said drugs

## 5 1. Field of the invention

The present invention relates to the use of F(ab')<sub>2</sub> fragments and corresponding IgG immunoglobulins, active as specific antibodies to drugs and metabolites thereof, in the detoxification of humans and animals from said drugs.

10 Detoxification (i.e. removal of exogenous substances from organism) is extremely useful to neutralize the toxic effects that are induced, e.g., by drug overdose, accumulation, poisoning and, in man, also by drugs abuse.

It is of particular importance in the zootechnical field because, in  
15 the latest years, the search for ever new production goals and the growing development of intensive commercial-scale breeding spurred the recourse to drugs, whereby the traditional production rhythms are deeply affected.

Several medicinal substances are used in zootechny, e.g., (i) to  
20 prevent and treat infectious diseases, in particular the zoonotic ones, i.e., diseases or infections common to animals and humans and mutually communicable, representing about half of the over four hundred infections or infestive diseases, which are a prospective risk for livestock; (ii) to prevent infestations; (iii) to improve the  
25 growth rate and increase food utilization; (iv) to promote milk production.

Out of the drugs used in the zootechnic field to enhance livestock

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performance, i.e., increase the assimilation rate and favour a more complete food utilization, the auxinic, anabolic drugs, anti-thyroid agents, cortisone, corticosteroids and their synthetic analogs, and sympathicomimetic drugs are the most widely used.

- 5 Out of the auxinic drugs, mention is made of virginiamycin, zincobacitracin, avoparicin antibiotics, which act on the microflora of the alimentary canal and improve food assimilation.

- Out of the anabolic drugs, mention is made of native endocrine hormones, the most widely used in zootechny being 17- $\beta$ -estradiol, 10 testosterone, progesterone, exocrine hormones obtained by synthesis, the most widely used in zootechny being 19-nortestosterone (nandrolone), and trenbolone, which are androgenic, and zeranol and diethylstilbestrol (DES) which are estrogenic.

- Among anti-thyroid treating agents, the most widely used are 15 methylthiouracil and propylthiouracil.

- Cortisone and related drugs endowed with corticosteroid activity, hereinafter referred to as corticosteroid analogs, act on the glucidic and protidic metabolism and cause retention of fluid; they can be combined with endogenous hormones as low-absorption dexamethazone 20 based compositions.

The most widely used sympathicomimetic drugs are  $\beta$ -agonist clenbuterol and cimaterol, which favour lipolysis and proteic synthesis.

Finally, somatotropin is used to enhance growth and milk production.

- The advantages obtained in terms of simplicity of use and results 25 achieved are undeniable. However, there are a series of risks for a consumer, due to the presence of medicaments or of their active metabolites in the organism or in milk and meat derived from treated

animals. Therefore, a sufficient time for total drug elimination from the organism has to elapse before animal slaughtering or milk collection to safeguard consumers from the potential risk of the side effects thereof, which is particularly high in low-bodyweight  
5 subjects, such as sucklings and babies.

The lack of rapid and accurate diagnostic methods useful to detect circulating levels of drugs and metabolites thereof to be performed directly at farms further complicates the possibility of an *ad hoc* therapeutic intervention and, in some cases, favours a great number  
10 of drugs or higher dosage rates to be administered.

## 2. Technical problem

It follows that there is an urgent need for methods of control of the presence of circulating medicinal or toxic substances as well as for an as rapid as possible method of their elimination, not involving  
15 further risks for the subject treated and, in case of livestock, for the consumer of zootechnical products derived therefrom.

## 3. State of the art

The immunoglobulino-therapy (serotherapy) of infectious diseases is known in clinical practice: gamma-globulins are administered (passive  
20 immunization), optionally in combination with active immunization.

## 4. Summary

The Applicant has surprisingly found that drugs and/metabolites thereof can be more rapidly eliminated from human and animal bodies by administering F(ab')<sub>2</sub> fragments of IgG immunoglobulins, active as  
25 specific antibodies to said medicaments or drugs and metabolites.

According to the present invention, drugs or metabolites, (haptens) which are unable by themselves to stimulate an immune response from

the body, are converted, by conjugation with heterologous proteins or polysaccharides, into immunogen derivatives, i.e., capable of stimulating the formation in the treated animals of IgG immunoglobulins active as specific antibodies to said drugs or  
5 metabolites.

IgG immunoglobulins obtained from animal serum are used to prepare  $F(ab')_2$  fragments by removal of the Fc fragment; they may also be used as are for detoxifying treatments and as diagnostic reagents in a method of qualitative or quantitative immunodiagnostic determinations  
10 of the concentration in a biological fluid, of a drug and/or metabolites thereof, whereto they act as specific antibodies.

The present invention relates to the aforesaid  $F(ab')_2$  fragments and IgG immunoglobulins, the pharmaceutical compositions containing same, the method of their preparation, and their use for the detoxification  
15 of humans and animals.

The invention also includes the aforesaid immunogen derivatives and the preparation process thereof; the aforesaid immunodiagnostic method and the corresponding diagnostic kit; drug-enzyme conjugates useful as diagnostic reagents for the aforesaid diagnostic method and the method  
20 of their preparation, as disclosed in the present text.

The  $F(ab')_2$  fragments of the invention enhance the *in vivo* elimination of drugs and/or metabolites from the body, presumably by forming antigen-antibody complexes which promote macrophages opsonization and phagocytosis, with subsequent increase in elimination.

## 25 5. Detailed description of the invention

The molecular weight of the claimed IgG immunoglobulins is of about 150,000 Daltons; that of the corresponding  $F(ab')_2$  fragments is lower



[e.g. of about 100,000 daltons].

The claimed  $F(ab')_2$  fragments find extensive application in the detoxifying treatment of drug-treated humans and animals, e.g., in case of drug poisoning, overdose, for instance in humans in case of  
5 abuse of pharmacologically active substances.

The claimed  $F(ab')_2$  fragments find in particular application in the zootechnical field in the detoxification of drug-treated livestock (e.g. mammals such as cattle, sheep, pigs) before slaughtering or milk collection.

10 The removal of Fc fragments from the present IgG immunoglobulins allows them to be despeciated and to increase organism's tolerance towards them. In any case, the detoxifying treatment is well tolerated also when the present IgG immunoglobulins are administered to animals belonging to the same species as that utilized for their production by  
15 immunization.

The present detoxifying treatment is, e.g., meant for promoting the elimination of the following drugs from the organism:

- antibiotics, e.g., tetracyclines (such as oxytetracycline, doxycycline); amino glycosidic antibiotics (such as neomicyn B,  
20 gentamicin C, tobramycin, kanamycin, amikacin, and streptomycin; cephalosporins (e.g. ceftazidime, cefotaxime, cephalexin); penicillins;
- sulphonamides (derived from sulphanilamide, e.g., substituted with an isoxazole ring at the sulphonamide nitrogen, such as  
25 sulphamethoxazole and sulphamethazine;
- antiviral drugs, such as idoxuridine;
- antiparasitic drugs;

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- antimycotic drugs (amphotericin, ketoconazole);
- antitubercular drugs, such as cycloserine, isoniazid;
- sympathicomimetic drugs, in particular having 2-phenylethylamine structure, e.g.,  $\beta$ -agonist such as clenbuterol and cimaterol;
- 5 - anti-thyroid treating agents, in particular 2-thiouracils (e.g. methylthiouracil and propylthiouracil);
- estrogenic substances, used, e.g., as anabolic steroids in zootechny, such as diethylstilbestrol, 17- $\beta$ -estradiol, zeranol;
- androgenic substances, used, e.g., as anabolic steroids in
- 10 zootechny, such as trenbolone, 19-nortestosterone (nandrolone);
- progestinic substances (progesterone);
- cortisone and corticosteroid analogs, e.g. adrenocorticosteroids, such as dexamethasone;
- other endogenous hormones, such as somatotrophin (growth hormone
- 15 STH);
- auxinic drugs, out of which antibiotics, such as virginiamycin, zincobacitracin, avoparcin;
- amphetaminic drugs (amphetamine, methamphetamine);
- growth stimulants, e.g. with quinoline structure such as olaquinox;
- 20 - disinfectants.

According to a typical embodiment of the present invention, the claimed antibody F(ab')<sub>2</sub> fragments (or the claimed IgGs) are administered to the subject to be detoxified by the parenteral route, in particular by the intramuscular (i.m.), subcutaneous (s.c.),

25 intravenous (i.v.) or intradermal (i.d.) route, typically by the i.m. or i.d. route.

Preferably, prior to their administration, the hematic, serum or milk

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concentration of the drug to be eliminated is determined to ascertain more accurately the dose of immunodetoxifier to be administered.

To obtain a substantial drug elimination within a short time (e.g. within 24 to 48 h),  $F(ab')_2$  are preferably administered at a dose  
5 corresponding to  $F(ab')_2$ : drug or metabolite molar ratios for instance comprised between 1:1 to 1:5, referred to drug or of metabolite present in the blood of the subject to be treated [each mol of  $F(ab')_2$  fragment or of IgG can bind a molar amount of drug equal to or higher than 1 mol of drug].

10 When integral IgG are used, they can be administered at a dose of 1 mole of immunoglobulin per 10 or more than 10 mole of drug or metabolite present in circulating blood, depending on the antibody's (immunoglobulin's) avidity.

Should drugs and/or metabolites be still present in the blood, said  
15 first  $F(ab')_2$  (or IgG) administration will be followed, if so required, by one or more further administrations.

In particular, the determination of the drug and/or metabolite concentration in the blood of the subject to be treated is to be repeated about 24 to 72 h after each administration of the claimed  
20  $F(ab')_2$  fragments (or IgG) to check whether some drug and/or metabolites thereof are still present in the blood.  $F(ab')_2$  fragments (or IgGs) according to the present invention are typically administered in the form of pharmaceutical compositions including at least one of the aforesaid fragments (or IgGs), in a therapeutically  
25 effective amount, combined with one or more pharmaceutically acceptable excipients or diluents, either singly or as a mixture thereof, and may, e.g., be prepared by conventional methods, like

those reported in Remington's Pharmaceutical Sciences, 18th Ed., 1990. Particularly preferred are the preparations for injection, comprising at least one of the aforesaid fragments (or IgGs), at least a diluent (e.g., a solvent, such as physiological saline solution, wherein they  
5 are dispersed, dissolved or emulsified), and other excipients, if any. The preparations for injection may be in the form, e.g., of two vials, one enclosing the  $F(ab')_2$  fragments (or IgGs) in the solid state and the other enclosing the diluent or solvent.

Pharmaceutical composition can be also in the retard form.

- 10 Preparation of  $F(ab')_2$  fragments: they are obtained by removing by digestion with pepsin the  $F_c$  fragment of the corresponding IgG immunoglobulins obtained from the serum of animals treated with immunogen derivatives, these last being obtained by conjugating the compound selected as the hapten for the drug to be removed from the  
15 body with a proteic or polysaccharide carrier.

#### Preparation of immunogen derivatives

The carrier used for the preparation of immunogens is typically a heterologous protein i.e. derived from an animal of a species differing from that used for the production of IgG antibodies, or a  
20 polysaccharide. Exemplary proteins that may be used as carriers are bovine serum albumin (BSA), human serum albumin (HSA), chicken ovoalbumin (OVA), bovine globulins, chicken or turkey globulins, limset hemocyanin. Bovine or human serum albumins, chicken ovoalbumin and gamma-globulins are preferably used to produce antibodies in  
25 animals, such as rabbits, goats or horses.

Hapten: proteic carrier molar ratios found in the immunogens or used for immunogen preparation depend on the proteic carrier molecular

weight.

Hapten: proteic carrier molar ratios founds in the immunogens range for instance about from 15:1 to 50:1, e.g. about from 15:1 to 20:1 in the case of HSA or BSA, and in general of proteic substances with  
5 molecular weight of about 40,000-70,000 daltons (included those used for conjugation for diagnostic purposes), and are about proportionally higher in the case of higher molecular weight proteins, e.g. up to 2-3 folds higher in the case of IgGs having M.W. 160,000 daltons.

Unless otherwise reported, hapten is typically added to the proteic  
10 substance both for the preparation of immunogens or of diagnostic conjugates) in further excess with respect to that found in the final product; the hapten: proteic carrier molar ratios added to the reaction mixture range for instance about from 100:1 to 200:1 for HSA or BSA, which has a molecular weight of about 65,000 daltons; e.g.  
15 from about 20:1 to 60:1 for OVA.

The unreacted hapten excess is suitably removed from the reaction mixture, e.g. by dialysis.

Proteic or polysaccharidic carriers have for instance molecular weights ranging from 40,000 to 450,000 Daltons (for example, BSA  
20 having a MW of 65,000).

The compound conjugated with the carrier to produce an immunogen of said compound is called hapten. The hapten preferably utilized is the substance to be eliminated from the organism, and can be the same drug administered to the subject to be treated, a metabolite thereof, if  
25 said metabolite is the active compound to be eliminated from the organism, or a structurally related derivative thereof, e.g., a derivative thereof, functionalized with functional groups suitable for

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the covalent conjugation with the proteic or polysaccharide carrier to be obtained.

For example, the hapten preferably used for cephalosporins is the same cephalosporin administered to the subject to be detoxified. Should  
5 the drug to be eliminated be a penicillin, the hapten preferably used will be the metabolites thereof, 6-aminopenicillanic acid (6-APA) or penicilloic acid.

The hapten preferably used for tetracyclines is the same drug administered, preferably functionalized as illustrated in the present  
10 text.

Examples of tetracyclines used as drugs and as haptens are chlortetracycline, oxytetracycline, demethyltetracycline, methacycline, doxycycline, and minocycline.

The hapten preferably used for amino glycosidic antibiotics is  
15 preferably the specific antibiotic administered to the subject to be detoxified.

The hapten preferably used for sympathicomimetic drugs having 2-phenylethylamine structure is the same drug administered to the subject to be detoxified.

20 As concerns cephalosporins, penicillins, tetracyclines and sympathicomimetic drugs having 2-phenylethylamine structure and particular examples thereof, reference is made to Goodman & Gillman, "The pharmacological basis of therapeutics", The Macmillian Co., 3rd Ed., 1966, pp. 1194-1227, in particular p. 1195, pp. 1242-1257, p.  
25 484, and to the translation into Italian of the 6th American edition of Goodman and Gilman's book: "Le basi farmacologiche della terapia", Editoriale Grasso, Bologna, 1982, pp. 1234-1239 and p. 1265.

The hapten typically used for sulphonylamides is the same compound administered to the subject to be detoxified, or a sulphonamide substituted at the  $-SO_2NH_2$  sulphonamide nitrogen with an aromatic ring of the same type as that present in the drug. For example, for  
5 sulphamethoxazole, sulphamethoxazole itself or another sulphonamide containing an optionally substituted isoxazole ring is used.

The hapten preferably used for anti-thyroid 2-thiouracyl derivatives is a 2-thiouracyl derivative substituted at position 6 with a  $C_1-C_3$  alkyl, preferably functionalized as illustrated in the present text.

10 The hapten preferably used for diethylstilbestrol, zeranol or trenbolone is the same drug administered, preferably functionalized as hereinafter illustrated. The hapten preferably used for 17- $\beta$ -estradiol and derivatives thereof (e.g. esters, such as estradiol 17- $\beta$ -cypionate and estradiol 3,17- $\beta$ -dicypionate) is 17- $\beta$ -estradiol, preferably  
15 derivatized as hereinafter described.

The type of carrier and hapten-carrier conjugation depends on the chemical structure of the drug and/or metabolite to be converted into immunogen. Conjugation generally takes place by formation of one or more covalent bonds between the hapten and the carrier.

20 Some methods of preparation of immunogens are reported below by way of illustration.

Immunogen derivatives represented by hapten/proteic carrier conjugates obtained by treating a hapten with a proteic carrier in the presence of a carbodiimide

25 The method is suitable for the conversion into immunogens of haptens containing  $-NH_2$  aliphatic or aromatic primary amino groups, or  $-COOH$  carboxy groups.

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The -COOH groups of the proteic carrier (or hapten), activated by carbodiimide reaction through formation of the corresponding O-acylisourea, react with the -NH<sub>2</sub> groups of the hapten (or proteic carrier) with formation of an amide bond.

- 5 The proteic carrier can be e.g. BSA, HSA, OVA or IgG, for instance HSA or OVA for method A, and gamma-globulin IgG for method B, as hereinafter illustrated; the carbodiimide is preferably N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide or another pharmaceutically acceptable carbodiimide, giving pharmaceutically acceptable reaction
- 10 products (e.g., dialkylcarbodiimides containing C<sub>1</sub>-C<sub>12</sub> alkyl groups, optionally substituted with a tertiary amino group, such as -N(CH<sub>3</sub>)<sub>2</sub>).

Method A - (Carbodiimide direct conjugation method) - Immunogen derivatives obtained by conjugation of a hapten directly with a proteic carrier, in the presence of a carbodiimide, for instance by

15 preactivation with carbodiimide of the compound containing the -COOH groups.

The hapten/proteic carrier conjugate is obtained, e.g., by causing the compound containing -COOH groups (e.g., the proteic carrier, in case of haptens containing NH<sub>2</sub> groups or a hapten containing -COOH groups)

20 to react with A carbodiimide in a stoichiometric excess with respect to the -COOH groups, typically in an aqueous medium, generally at an alkaline pH (e.g., 8-10), at about 10°C to 40°C (e.g., 37°C). The resulting product is treated with a compound containing -NH<sub>2</sub> groups (the hapten containing -NH<sub>2</sub> groups, or the proteic carrier, if a

25 hapten containing -COOH groups has been activated with carbodiimide). According to some specific embodiments of the present invention, hapten, proteic carrier and carbodiimide, e.g. N-ethyl-N'-(3-



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dimethylaminopropyl)-carbodiimide, are for instance reacted in aqueous medium, e.g. at pH 5.0-6.0. e.g. 6.0, with excess carbodiimide, at a temperature e.g. of +30°C, e.g. at +10°C.

Hapten: proteic carrier molar ratios reacted for the preparation of  
5 immunogen can be for instance of from 100:1 to 200:1 for BSA, HSA and from 20:1 to 60:1 for OVA.

In any case, carbodiimide condensation may be carried out according to the method disclosed by E.C. Beuvery, G.J.A. Speijers, B.I.G. Lutz, D. Freudenthal, V. Kanhai, B. Haagmans, and H.J.G.M. Derks, in Develop.  
10 Biol. Standard, S. Karger, Basel (1986), 63, 117.

Method B - Immunogen derivatives obtained by conjugation, in the presence of a carbodiimide, of a hapten containing primary -NH<sub>2</sub> groups, with a proteic or polysaccharidic carrier previously oxidized and conjugated with a poly-amino acid copolymer.

15 According to a variant developed by the Applicant, the hapten is conjugated with a proteic or polysaccharidic carrier, e.g. with a glycoproteic carrier, through a bridge of a poly-amino acid copolymer, such as Poly.(D-Glu, D-Lys). The relevant procedure, described hereinafter in greater detail with reference to a proteic carrier  
20 substance, comprises the step of:

- a) treating a proteic substance with an oxidizing agent of the -C-OH groups to C=O groups; ✓
- b) treating the resulting oxidized proteic substance with a synthetic poly-amino acidic copolymer, and the product thus obtained with a  
25 reducing agent, such as NaBH<sub>4</sub>;
- c) treating the resulting oxidized proteic substance/amino acid copolymer conjugate with the hapten, in the presence of a

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carbodiimide.

In the present text, the -C-OH groups are alcoholic groups and the oxidizing agent is more particularly an oxidizing agent of primary alcoholic groups to aldehydic -CHO groups.

- 5 An advantage offered by this method is the possibility of controlling accurately the amount of hapten that links to the proteic carrier (which is still found in the final product in an amount substantially corresponding to the amount caused to react).

In immunogens preparation, the proteic substance is the carrier and  
10 the hapten is the drug or the metabolite thereof to be detoxified, or the derivatives thereof selected as a hapten for said drug.

According to method B, the proteic carrier can have molecular weight 40,000-70,000, such as BSA, HSA with molecular weight 65,000, or can be a glycoprotein, for instance a gamma-globulin IgG (for instance  
15 with a molecular weight of 160,000 Daltons). Alternatively, the carrier can be a polysaccharidic polymer with molecular weight comprised between about 50,000 and 200,000 Daltons, such as for instance dextrans or alginates.

Oxidation of the proteic substance is typically carried out with  
20 periodate, typically an inorganic periodate, e.g. in water, at a pH preferably ranging from 4.5 to 7.5, preferably at +4°C to 40°C, and shielded from light.

Oxidation is typically carried out with  $\text{NaIO}_4$ , at a pH of 5.0 (e.g., in acetate buffer), at room temperature (about 20°C to 25°C).

- 25 The oxidizing agent is typically in a stoichiometric excess with respect to the -OH groups of the proteic substance, preferably of 0.01M-0.1 moles of  $\text{NaIO}_4$  each  $10^{-7}$  -  $10^{-8}$  moles of proteic substance,

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e.g. of glycoproteic carrier.

The reaction is generally complete after about 15-60 min and the excess of oxidizing agent is generally removed by addition of molar excess of alcohol, typically glycerol or ethylene glycol, at the  
5 abovementioned oxidation temperature. Before performing the successive step, the reaction mixture is generally allowed to stand at 0°C/+5°C to allow excess  $\text{NaIO}_4$  to react with the alcohol.

The poly-amino acid copolymer is typically a copolymer containing acid  $\alpha$ -amino acids (which contain at least a further -COOH group  
10 or another acidic group in addition to the  $\alpha$ -carboxy group) and basic  $\alpha$ -amino acid (which contain other  $-\text{NH}_2$  groups or other basic groups in addition to the  $\alpha$ -amino group). The copolymer has a molecular weight for instance of 20,000-100,000 daltons, preferably 50,000, and contains L or D amino acidic residues, or mixtures thereof. It  
15 contains different aminoacidic sequences, in different ratios, provided that it contains glutamic acid (Glu) and Lysin (Lys).

Preferably, a D-Glu,D-Lys copolymer having a MW typically ranging from about 20,000 to 100,000, preferably from 20,000 to 50,000 is used, such as the Poly(D-Glu,D-Lys) copolymer having a MW of about 50,000  
20 and a Glu:Lys ratio equal to 6:4, available under the trademark Sigma R P7658.

Condensation b) between the oxidized proteic substance and the copolymer is generally carried out in an aqueous medium, at an alkaline pH, e.g., of about 8-10, generally at a temperature ranging  
25 from 4°C to 40°C, e.g. at 4°C to 8°C overnight (e.g., in a refrigerator), typically from 30°C to 40°C for about 2 to 4 h, and shielded from light. Typically, the condensation is carried out at a

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pH of about 9 (e.g. in  $\text{CO}_3^{--}/\text{HCO}_3$  buffer), at 37°C., for 2-4 hours.

The oxidized protein/copolymer molar ratio used in the reaction medium typically ranges from 1:5 to 1:30, e.g., 1:15, which is substantially found in the conjugate obtained.

- 5 The aforementioned reducing agent of step b) is typically  $\text{NaBH}_4$ , typically used in molar excess, e.g. at about 0°C to 10°C, generally at 4°C to 6°C for 2-6 hours, typically 4 hours.

This treatment with reducing agent has the scope of reducing the double bond ( $\text{HC=N-}$ ) formed during condensation according to step b),

- 10 allowing stabilization of the final compound.

The oxidized proteic substance/poly-amino acid copolymer conjugate so obtained is typically purified by dialysis on membranes with molecular exclusion limit (cutoff) typically ranging from 200,000 to 300,000, e.g. about 200,000 Daltons (200 KD), against a buffer at a pH of about  
15 5.0 to 6.0, e.g., 5.5, and preferably at cool temperature, at about 0°C to 10°C, generally at 4°C to 8°C. Separation from non-oxidized protein residue is also obtained.

The condensation between the hapten and oxidized carrier, conjugated with the copolymer [phase c)], is typically carried out with N-ethyl-  
20 N'-(3-dimethylaminopropyl)-carbodiimide.

The condensation is typically carried out in an aqueous medium, at a pH preferably ranging from 5.0 to 6.0 (e.g., 5.5, in acetate buffer), e.g. at about 30°C to 40°C, generally at 37°C. For instance, aqueous solutions of hapten and proteic substance/poly-amino acidic copolymer  
25 conjugate are mixed (e.g., in the concentrations reported in the Experimental Part), carbodiimide is added and the reaction temperature is brought to the selected value.

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The hapten added to the proteic substance substantially corresponds to the molar ratio hapten: proteic substance found in the final conjugate, which is preferably of about 15:1 to 50:1, typically 15:1 in respect of the poly-amino acidic copolymer present in the proteic substance/poly-amino acidic copolymer conjugate.

The amount of copolymer substantially corresponds to the amount added to the oxidized proteic substance, as proved by proteic content determination and electrophoretic analysis carried out according to conventional techniques.

Carbodiimide is typically used in a molar excess with respect to the -COOH groups of the copolymer-protein conjugate, e.g., of about 3 - 6 mmol per 1-10  $\mu$ mol of protein/amino acidic copolymer conjugate, these molar ratios being in particular referred to a conjugate between a glycoprotein carrier with a M.W. in the range 65,000-200,000 daltons and a poly-amino acid copolymer with a M.W. of about 50,000 daltons.

The hapten/carrier conjugate is typically purified by dialysis, by causing it to pass through membranes with molecular exclusion limit (cutoff) value of about 10,000 to 300,000 Daltons, depending on molecular weight of the copolymer and of the protein, preferably of about 12,000 Daltons, against a buffer at a pH of about 6.0 to 8.0, preferably of 7.0 to 7.5, e.g., against PBS (physiological saline solution/phosphate buffer), operating preferably at 4°C to 10°C (e.g., about 4°C). Low-molecular-weight fragments and salts are thus eliminated.

Conjugation of haptens with carriers in the presence of carbodiimide applies to haptens with primary amino groups -NH<sub>2</sub> or with -COOH groups, direct conjugation according to method A) being for instance

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applied to haptens with  $\text{-COOH}$  or  $\text{-NH}_2$  groups, and conjugation via poly-aminoacid copolymer bridge (i.e. method B) being for instance applied to haptens with primary amino groups  $\text{-NH}_2$ .

The immunogen is the conjugation product through one or more covalent  
5 bonds between the hapten and the proteic carrier, either as such or as oxidized glycoproteic carrier/poly-amino acid copolymer conjugated, for instance:

- amide bonds between  $\text{-COOH}$  groups of haptens and  $\text{-NH}_2$  groups of proteic carriers, according to the direct method proteic carrier-  
10 haptens, as for instance for cephalosporins and penicillins metabolites;

- amide bonds between  $\text{-NH}_2$  groups of haptens and  $\text{-COOH}$  groups of the glycoproteic carrier, according to the method carrier-copolymer-hapten (Method B.), as for instance for aminoglycosides, cycloserine,  
15 amphetamine, sulphamethoxazole.

Conjugation in the presence of carbodiimide either with a proteic carrier as such (Method A), or with a glycoprotein oxidized and conjugated with an poly-amino acidic copolymer as described above as to Method B, is applied, e.g., to the conversion into immunogens of  
20 the following compounds:

Method A [protein-hapten *via* carbodiimide direct conjugation method; haptens react with their  $\text{-COOH}$  groups] is in particular suitable for:

- cephalosporins, for instance ceftazidime, cefotaxime, cephalixin (cephalosporins typically react with the  $\text{-COOH}$  at position 4);  
25 - 6-amino penicillanic acid and penicilloic acid, selected as haptens for the preparation of  $\text{F(ab')}_2$  active as antibodies to penicillins.

Method B [conjugation protein-hapten-copolymer, preferably glyco-

protein-hapten-copolymer] is in particular suitable for conversion into immunogens of the following compounds containing primary aliphatic or aromatic groups:

- amino glycosidic antibiotics, e.g. neomycin, gentamicin, streptomycin;
- antimycotic drugs, e.g. amphotericin;
- antitubercular drugs, e.g. cycloserine;
- sulphonylamides, e.g. sulphametoxazole;
- amphetamine;
- clenbuterol, cimaterol.

Drugs or metabolites can be functionalized so to allow at least one -COOH group to be introduced in their molecule. For instance, drugs or metabolites containing aromatic groups activated towards diazotization (i.e. towards electrophilic substitution with diazonium reagents) e.g.

- containing one or more phenolic groups, such as

- tetracyclines, such as oxytetracycline;
- steroids or analogs thereof containing at least one phenolic group -OH, e.g. 17-beta-estradiol, diethylstilbestrol, zeranol,
- or compounds with a quinoxaline ring, such as olaquinox,

- can be converted into reactive haptens, by reacting them with diazotized para-amino benzoic acid, which affords the corresponding 4-carboxyphenyl azo derivative, thus allowing a -COOH group to be introduced in the drug molecule (for instance according to the procedure of N. Weliky, H.H. Weetall, Immunochemistry, 2, 293, 1965), then conjugated with a carrier, for instance with BSA, HSA or OVA in the presence of a carbodiimide, according to Method A.

Examples are the conjugation product of HSA with 5-azo-(4-

carboxyphenyl)-N-(2-hydroxyethyl)-3-methyl-2-quinoxaline carboxamide (i.e. the reaction product of olaquinox with diazotized para-amino benzoic acid), and the conjugation product between HSA and the reaction product of oxytetracycline with diazotized para-amino benzoic  
5 acid, both obtained in the presence of carbodiimide, such as the conjugates according to examples 9d and 10d, respectively.

Drugs or metabolites with keto groups can be reacted with compounds containing both a carboxylic group and a function able to react *via* nucleophilic attack with the keto group (such as hydroxylamines or  
10 hydrazines carrying also carboxy groups, either as such or masked), thus allowing a carboxy group to be introduced into the molecule, then conjugated with a carrier, e.g. with BSA, according to Method A.

For instance, compounds with keto groups can be reacted with O-(carboxymethyl)-hydroxylamine [i.e. carboxymethoxylamine or aminoxy-  
15 acetic acid], following e.g. to the procedure of R. J. Warren and Fotherby, J. Endocrinol., 1974, 62, 605, thus allowing a carboxy group to be introduced into the hapten molecule, and the corresponding O-(carboxymethyl)oxime derivatives thus obtained are then conjugated with a carrier, e.g. with BSA, according to Method A: for instance,  
20 ketokonazole was converted into immunogen by this route.

Compounds with keto groups (e.g. trenbolone) can be for instance converted into the corresponding hydrazones containing a -COOH group by treatment with para-hydrazinobenzoic acid, following for instance the procedure of B. Africa, E. Haber, Immunochemistry, 8, 479, 1971,  
25 and the derivatives thus obtained are conjugated with a carrier, e.g. with BSA, according to Method A.

Secondary amines, for instance aliphatic ones such as methamphetamine,



can be for instance converted into reactive haptens by reaction with N-(4-bromobutyl)phtalymide, e.g. following the procedure of G.W. Aherne, E.M.Piall, V. Marks, in Br. J. Clin. Pharmacol., 3, 561, 1976, then hydrolizing the phtalymido group to give the corresponding  
5 primary amines, i.e. the corresponding primary amine with the 4-aminobutyl residue linked to the hapten secondary nitrogen, which are then reacted with the -COOH groups of a proteic carrier, e.g. with BSA, according to Method A.

Method C - Immunogen derivative obtained by conjugation of a proteic  
10 carrier with a hapten having -C-OH- groups oxidized to C=O groups,  
followed by reduction with NaBH<sub>4</sub>.

Amino glycosidic antibiotics and other drugs containing -C-OH groups in a glucydic moiety, such as neomycin, gentamicin, streptomycin, the antimycotic drug amphotericin, and idoxuridine are suitably converted  
15 into immunogens by treatment with an oxidizing agent capable of oxidizing the -C-OH groups to C=O groups, followed by treatment with a proteic carrier, e.g. BSA, HSA then by treatment with NaBH<sub>4</sub>.

Hapten oxidation is typically carried out with excess periodate, such as NaIO<sub>4</sub>, in an aqueous medium, at a pH preferably ranging from 5.0 to  
20 7.5, e.g., at a pH of 5.5 (e.g., in acetate buffer), at temperatures generally ranging from 4°C to 40°C preferably at room temperature (+20°C/+25°C) or at 37°C, and shielded from light. The excess of oxidizing agent is typically neutralized by glycerol or ethylene glycol addition, as described above for the oxidation of proteic  
25 carriers; the reaction of oxidized hapten and protein (typically BSA or HSA having a MW of 65,000) is typically carried out at an alkaline pH (preferably of about 8.5 to 9.5) at the oxidation temperature

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specified above.

Method D - Immunogen derivative obtained by conjugation of a hapten with a proteic carrier with -C-OH groups oxidized to C=O groups followed by reduction with NaBH<sub>4</sub>

- 5 The method concerns the preparation of hapten/proteic carrier conjugates, of haptens containing primary -NH<sub>2</sub> groups, e.g., amino glycosidic antibiotics, amphotericin, and proteic carriers oxidized with oxidizing agents capable of oxidizing the -OH groups to C=O groups.
- 10 The proteic substance (a carrier in the case of immunogens) is typically oxidized with periodate, under the aforementioned conditions (step a). The excess oxidizing agent is eliminated by glycerol addition as described above. The successive conjugation with the hapten is typically carried out at an alkaline pH (e.g., of about
- 15 8.5 to 9.5), at temperatures generally ranging from 4°C to 40°C (e.g., at 4°C to 8°C overnight or at 20°C to 37°C for about 2 to 6 h) and is followed by treatment with NaBH<sub>4</sub>. The proteic carrier can be for instance BSA (e.g. having a MW of 65,000), or HSA and is oxidized with periodate; the drugs converted into immunogens are, e.g.,
- 20 neomycin, gentamicin, streptomycin, and amphotericin.

Method E - Immunogens obtained by conjugation of the hapten with a proteic carrier in the presence of glutaraldehyde

- The method is suitable for the conjugation of haptens containing primary aliphatic or aromatic -NH<sub>2</sub> amino groups with proteic
- 25 substances.

Under typical conditions, glutaraldehyde is added to a mixture of proteic substance and hapten, in an aqueous medium, at a pH generally

ranging from 6.0 to 8.0, preferably from 6.7 to 6.9 (e.g., in phosphate buffer), at about 4°C to 40°C, typically at room temperature (20°C to 25°C).

The reaction is complete in about 0.1 to 5 h, typically in 3 hours.

- 5 Glutaraldehyde is typically used in stoichiometric excess with respect to the  $\text{-NH}_2$  groups of the proteic carrier, for instance in amounts of  $10^{-4}$  to  $10^{-5}$  moles each  $10^{-5}/10^{-6}$  moles of  $\text{-NH}_2$  groups of the proteic substance (e.g. BSA has about 59 moles of  $\text{-NH}_2$  groups per mole of BSA). For instance, glutaraldehyde is used in an amount ranging from
- 10 0.1 to 1.0 mg, e.g., 0.5 mg per mg of proteic substance. Once the reaction is complete, excess glutaraldehyde is neutralized by treatment with lysine which is typically used in an amount of about 1.5 to 15 mg, e.g., 7.5 mg per mg of proteic carrier), under the same operating conditions used for the conjugation with the hapten.
- 15 The hapten/proteic substance molar ratio preferably ranges from 10:1 to 50:1, and more preferably is equal to 20:1.

For immunogens preparation, the proteic substance is for instance BSA (e.g., having a MW of about 65,000 Daltons) or HSA.

- The resulting hapten/proteic substance conjugates, and more generally
- 20 all hapten/proteic substance conjugates described herein, are preferably purified by dialysis, typically through membranes with molecular exclusion limit (cut-off) of about 10,000 daltons (10 KD), preferably against a buffer with pH 6.0-8.0, for instance pH 7.0-7.5, such as PBS (saline solution) phosphate buffer). The conjugation in
- 25 the presence of glutaraldehyde may take place, e.g., according to the method described by S. Avrameas, Immunochemistry, Pergamon Press, 1969, 6, 43-52 [cited by N. Zegers, K. Gerritse, C. Deen, W. Boersma,

and E. Classen, in J. Immunol. Meth., 130, 195 (1990)].

For instance, in the present invention the glutaraldehyde method has been in particular adopted for the conversion of the following haptens into immunogens:

- 5 - sympathicomimetic drugs, which contain aromatic  $-NH_2$  groups, such as cimaterol, clenbuterol;
- amino glycosidic antibiotics, e.g. neomycin, gentamicin, streptomycin;
- antimycotic drugs, e.g. amphotericin;
- 10 - amphetamine;
- antitubercular drugs, e.g. cycloserine;
- sulphonylamides, e.g. sulphamethoxazole.

Method F - Conjugation of haptens containing thiol groups with a proteic carrier, previously functionalized with a disulfide reagent

- 15 Immunogens of haptens containing one or more thiol groups can be for instance obtained by conjugating them with proteic carriers, typically BSA, via a disulfide bridge, e.g. following the procedure described by D.A. Gadkari, H.A. Fields and J.E. Maynard, in J. Virol. Meth., 10, 215, (1985), or by T. Piao King, Y. Li, and L. Kochoumian,
- 20 in Biochemistry, 17(8), 1499 (1978). Accordingly, haptens containing one or more  $-SH$  groups, for instance 2-thiouracils such as propylthiouracil, can be conjugated with a proteic carrier, typically BSA or HSA, which has been previously treated with a reagent able to functionalize the proteic carrier with disulfide bonds, i.e. a
- 25 disulfide reagent, such as N-succinimyl-3-(2-pyridyldithio)propionate (SPDP) [i.e. 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester], which affords the corresponding 2-pyridyldithio-protein, or

with 4,4'-dithio-dipyridine (generally upon amidination, e.g. with 2-iminothiolane), which affords the corresponding 4-pyridyldithio-protein.

Both functionalization of the proteic carrier with the disulfide  
5 reagent and conjugation of the hapten with the derivatized proteic carrier can be for instance carried out at room temperature (e.g. at 23°C), at a pH generally of 7.0-7.5 (e.g. in PBS buffer, pH 7.4).

Hapten is reacted with the proteic carrier, typically BSA, e.g. in molar ratios with respect to proteic carrier typically of from 15:1 to  
10 50:1, e.g. 20:1 (essentially corresponding ratios are found in the conjugate).

The molar ratio between the dithio reagent and the protein can be for instance of about 3:1.

Method G - Conjugation of haptens containing aromatic groups reactive  
15 towards diazonium salts, with a proteic carrier, in the presence of a  
bis-diazo reagent

This method is suitable for instance for haptens such as isoniazide. The carrier is typically BSA or HSA, and the bis-diazo derivative is for instance bis-diazotized benzidine. Reaction medium, temperature,  
20 pH, and hapten: proteic carrier molar ratios can be for instance as hereinafter described for method H. The procedure of R.M. Bassiri, R.D. Utiger, in Endocrinology, 90, 722 (1972) can be for instance followed.

Method H - Conjugation of diazotized haptens with proteic carrier  
25 Haptens containing primary amino groups, in particular aromatic ones, such as sulphonyl amides, can be diazotized and coupled with a proteic carrier, e.g. BSA, HSA or OVA, for instance HSA.

According to some particular embodiment of the present invention, conjugation of the diazotized hapten with the proteic carrier is carried out in an aqueous medium, e.g. at pH of about 9.0-10.0, at a temperature e.g. of about +2°C/+5°C.

- 5 Diazotized hapten: proteic carrier molar ratios used to prepare the product can be for instance of from 100:1 to 150 to:1.

An example is the azo-hapten-protein conjugate obtained by reaction of diazotized sulphomethoxazine with HSA, such as that according to Example 11c.

- 10 Drugs and metabolites thereof can be converted into immunogens not only according to the methods cited in the present text, but also by other methods known in the immunological field for the preparation of conjugates of haptens (polysaccharides, polypeptides, toxins or other substances) with proteic (e.g. BSA) or polysaccharide (e.g., dextrane  
15 having a MW of 4,400) carriers, which are known for diagnostic or immunization (e.g., vaccines) purposes.

#### Production of IgG immunoglobulins

- The immunogen derivatives obtained as described above are administered to the animals selected for the production of IgGs active as specific  
20 antibodies, according to conventional techniques typically by a primary injection followed by a booster. Animal serums are then extracted and the proteic fraction corresponding to IgG immunoglobulins is isolated therefrom and purified.

- For commercial-scale production, the animals used are of medium/high  
25 size, such as goats and horses.

The claimed immunogen derivatives are preferably administered in combination with Freund's adjuvant. The method adopted is preferably

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that described by A. Johnstone and R. Thorpe, in *Immunochemistry in Practice*, p. 28, A. Johnstone and R. Thorpe, eds., Blackwell Sci. Publ., Oxford (1982).

In general, in the primary injection, the immunogen is combined with Freund's complete adjuvant (FCA) and, in the booster, with Freund's incomplete adjuvant (FIA). The primary injection is preferably administered by the intradermal route and the booster, typically administered 14 to 21 days later, by the intramuscular (i.m.) route. In both cases, each immunogen dose is generally of about 0.1 to 2 mg per kg of body weight, the first dose being preferably subdivided into several injections administered in different parts of the body.

IgG formation in the treated animal may be followed by passive hemoagglutination against the hapten covalently conjugated with turkey erythrocytes or by other conventional analytical methods.

About 30 to 90 days are required for the immunogen-treated animals to produce significantly high amounts of IgGs. After said period, a sample of blood is taken, generally from the femoral artery, wherefrom the proteic fraction containing IgG immunoglobulins is separated from serum, e.g., by precipitation by addition of ammonium sulphate.

The IgGs so obtained are preferably purified by ion-exchange chromatography, e.g. with matrix poly-[N-tris(hydroxymethyl) methylacrylamide functionalized with 2-(diethylamino)-ethyl groups, such as 1 M DEAE-Trysacryl (available from IBF), eluting with a buffer having a pH of 8.8. Typically, the cryoprecipitated serum containing IgGs is purified, and a preliminary passage on ULTROGEL AcA 202 column is performed for desalination.

The IgGs of the present invention may be purified according to other

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conventional techniques, e.g. by affinity by immobilization chromatography on protein A.

#### Conversion of IgG immunoglobulins into $F(ab')_2$ fragments

Fc fragments are removed by subjecting IgGs to digestion with pepsin, preferably immobilized, for instance under the solvent, pH and temperature conditions reported below.

Under typical conditions, the treatment with pepsin is carried out on proteic fractions precipitated by addition of polyethylene glycol, as disclosed by B. Favreau, D. Giurgiu, and B. Bizzini, in *Experientia*, 39, 483 (1983).

The process comprises of the following steps: precipitation of IgGs by treatment of the serum containing same with polyethylene glycol, typically at a pH of about 7.0 to 7.5, preferably of 7.0, at a temperature ranging from about 8°C to 25°C, e.g., at 20°C to 25°C; treatment of the resulting precipitate with pepsin, e.g., pepsin fixed on porous glass balls, at a pH of about 2.5 to 4.0, preferably of 3, at a temperature typically ranging from 25°C to 37°C, e.g., at about 30°C-32°C; precipitation of the fraction containing the  $F(ab')_2$  fragments by addition of polyethylene glycol, typically at a pH of about 4.5 to 5.5, e.g., of 5, for instance at room temperature (e.g. +20°C/+25°C).

The  $F(ab')_2$  fragments obtained are conveniently purified by dialysis, typically against 0.9% NaCl solution, overnight, at a temperature of 4°C to 10°C, e.g. at 4°C, and sterilized by filtration, usually through 0.45 µm filters.

#### Diagnostic process

The claimed IgGs are used as diagnostic reagents in an Enzyme Linked



Immunsorbed Assay (ELISA) of competitive type, e.g. analogous to that described by A. Voller, D.E. Bidwell, and A. Bartlett, in Bull. Wld. Health Org., p. 55, vol. 53 (1976).

Typically an inert solid support, is sensitized by fixation to IgG  
5 immunoglobulins active as antibodies to the compound to be proportioned, then added with the sample of biological fluid (e.g., serum, urine, milk) to be assayed, and then with a conjugate represented by the compound to be proportioned labelled with an enzyme, which is added either as a mixture with the sample to be  
10 assayed or a short time after the addition of said sample, after incubation, e.g. at 37°C, for about 30-60 minutes. The mixture is caused to react at 37°C for 30-60 minutes, preferably 60 minutes, to allow the fixation both of the compound to be proportioned and of the conjugate to the reactive site of IgGs; the mixture is washed and  
15 added with a chromogenic substrate which reacts with the labelling enzyme and, in the presence of the enzymatic portion of the conjugate, yields a coloured product. Upon colouring, the readout of the solution optical density is performed, in general directly in the proportioning microplate well, and the quantity of drug and/or metabolite contained  
20 in the assayed biological fluid is calculated.

Preferably, the enzyme is peroxidase, more particularly horse-radish peroxidase (HRP), and the chromogenic compound is ortho-phenylenediamine or tetramethylbenzidine (TMB), whose reaction with peroxidase is promoted by  $H_2O_2$ .

25 The drugs are, e.g., those listed herein for the claimed detoxifying treatment and/or as haptens for the preparation of immunogens.

The sample to be assayed is typically added in an amount sufficient

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for yielding a concentration of compounds to be assayed in a stoichiometric defect with respect to the IgG active sites: the compound to be proportioned and the enzyme-labelled compound compete for the linking sites of the specific antibody; the remaining sites  
5 are then bound to said conjugate, consequently part of the enzymatic activity is fixed to the solid support. The concentration of drug and/or metabolite to be proportioned is inversely proportional to the concentration of enzyme-labelled compound that is fixed to the antibody, and is calculated by difference, i.e., by subtracting the  
10 optical density of a sample in which IgG immunoglobulins have been completely saturated with the enzyme-labelled compound from the optical density of the IgG sample treated with the assayed biological fluid.

The solid support (e.g. a plate of plastic material, such as  
15 polystyrene) is sensitized with the IgG in a conventional manner, e.g., by treatment with the IgGs, at a pH of about 7.0-7.5, e.g. with 4 µg/100 µl IgG in PBS buffer at a temperature ranging from 4°C to 37°C (e.g., at 4°C to 8°C, overnight, or at 37°C for about 2 to 6 h), the sites of the solid support which failed to react with the IgGs are  
20 then saturated by successive addition of casein (e.g. a 2% casein solution in PBS) e.g. at a pH of about 7.0-7.5, at about 4°C to room temperature (e.g. +20°C/+25°C).

The reaction of the IgGs fixed to the solid support with the solutions to be assayed (sample solutions, enzyme-labelled solutions containing  
25 the drug or the metabolite, standard reference solutions) is typically carried out by incubation at about 20°C to 37°C, e.g., at 37°C, and at a pH of about 7.0-7.4, for 30-60 minutes, typically 60 minutes. Said

pH and temperature conditions may be adopted, e.g., also for colour development after chromogen addition, incubating for 30 minutes. As concerns washings and other operating details, reference is made to the Experimental Part.

#### 5 Diagnostic kit

The reagents useful for the diagnostic assay according to the present invention are advantageously assembled in an immunodiagnostic kit, to be directly used e.g., by the farmer. Said kit comprises an IgG immunoglobulin of the present invention supported on a solid phase, 10 the conjugate, represented by the enzyme-labelled compound to be proportioned (drug or metabolite), and a chromogenic substrate.

The kit may be also provided with buffer solutions for sample dilution and with standard solutions containing predetermined concentrations of the compound to be assayed.

#### 15 Conjugates represented by the enzyme-labelled compound to be proportioned (drug or metabolite)

Said conjugates are obtained by condensation of the compound to be proportioned (drug or metabolite) with an enzyme, in particular a peroxidase, for instance according to the aforementioned methods of 20 preparation of immunogen derivatives, wherein the hapten is the compound to be proportioned and the enzyme is the proteic substance to be used instead of the proteic carrier, or according to other known conjugation methods. For instance the compound to be proportioned can be reacted with the enzyme (peroxidase), in the presence of 25 glutaraldehyde, or with the SPDP method, or as described by A. Voller, D.E. Bidwell and A. Bartlett, in Bull. Wld. Health Org., 53, 55 (1976) in the presence of a carbodiimide.

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Exemplary conjugates useful as diagnostic reagents according to the present invention are obtained by conjugation of:

- a') a product obtained by conjugation of a hapten containing primary amino  $\text{-NH}_2$  groups or  $\text{-COOH}$  groups, with an enzyme in the presence of a carbodiimide;
- b') a product obtained by conjugation, in the presence of a carbodiimide, of a hapten containing primary amino  $\text{-NH}_2$  groups, with an oxidized enzyme/synthetic poly-amino acid copolymer conjugate, wherein the carrier has been previously oxidized with an oxidizing agent of the  $\text{-C-OH}$  groups to  $\text{C=O}$  groups;
- c') a product obtained by conjugation of an enzyme with a hapten containing  $\text{-C-OH}$  groups, previously oxidized with an oxidizing agent of the  $\text{-C-OH}$  groups to  $\text{C=O}$  groups conjugation being followed by reduction with  $\text{NaBH}_4$ ;
- d') a product obtained by conjugation of a hapten containing primary amino  $\text{-NH}_2$  groups, with an enzyme previously oxidized by treatment with an oxidizing agent of the  $\text{-C-OH}$  groups to  $\text{C=O}$  groups conjugation being followed by reduction with  $\text{NaBH}_4$ ;
- e') a product obtained by conjugation of a hapten containing primary amino  $\text{-NH}_2$  groups with an enzyme, in the presence of glutaraldehyde;
- f') a product obtained by conjugation of a hapten containing one or more thiol groups with an enzyme previously functionalized with a disulfide reagent;
- g') a product obtained by conjugation of a hapten containing one or more aromatic groups reactive towards diazonium salts, with an enzyme, in the presence of a bis-diazo reagent.
- h') a product obtained by conjugation of a diazotized hapten with an

enzyme.

Analogously with the considerations set forth for immunogens, the conjugates with enzymes of types a') and b') are particularly applied to drugs or metabolites containing  $-NH_2$ , or  $-COOH$  groups; the  
5 conjugates of types d') to compounds containing primary aliphatic or aromatic  $-NH_2$  groups. The considerations made for the analogous immunogens, i.e. concerning solvent, pH, temperature, molar ratios between reagents, and/or the poly-amino acidic copolymer, hold good also for the preparation of conjugates a') to g').

- 10 The enzyme is typically horse radish peroxidase having a molecular weight, e.g., of about 45,000.

Exemplary drugs or metabolites are those mentioned above for the detoxifying treatment and/or as haptens for the preparation of immunogens.

- 15 The following examples are conveyed by way of indication, not of limitation, of the present invention.

Method B - Conjugation of hapten, in the presence of carbodiimide, with a glycoprotein oxidized with periodate and conjugated with a poly-amino acid copolymer

- 20 a) 20 mg of proteic substance (glyco-proteic carrier for immunogen preparation or enzyme for drug/enzyme conjugates), having a MW of 40,000 to 200,000 Daltons, dissolved in 1.4 ml of 0.1 M acetate buffer, pH 6.5, was added with 200  $\mu$ l of  $NaIO_4$  0.1 M dissolved in the same buffer immediately prior to use. The mixture, shielded from  
25 light, was caused to react for 30 min at room temperature with gentle stirring. The reaction was discontinued by addition of 400  $\mu$ l of 1:1 glycerol/water, stirred for 5 min, allowed to stand for 4 h at 4°C.

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b) Reaction of oxidized protein and poly-(D-Glu,D-Lys) copolymer, Sigma<sup>R</sup> P7658 (having a MW of 50 KD); D-Glu:D-Lys ratio = 6:4; poly-(D-Glu,D-Lys):oxidized protein molar ratio equal to 1:15.

About 1.466 mg poly-(D-Glu,D-Lys) were necessary for 20 mg protein.

5 The reagents used were:

protein 20 mg in	2,000 µl acetate buffer
0,1M $\text{CO}_3^{--}/\text{HCO}_3^-$ buffer, pH 9.0	200 µl
Poly-(D-Glu,D-Lys) (2 mg/ml) in	733 µl carbonate buffer
0,1M $\text{CO}_3^{--}/\text{HCO}_3^-$ buffer, pH 9.0	267 µl

10 0.1 M carbonate buffer was added by adjustment of the reaction volume. The mixture, shielded from light, was caused to react at 37°C for 4 h, under stirring. Then at the end of reaction it was treated with 465 µl of  $\text{NaBH}_4$  (2 mg/136 µl of  $\text{H}_2\text{O}$ ), allowed to stand at 4°C for 4 h, still shielded from light. The resulting mixture was concentrated by  
15 centrifugation (3,000 rpm (rounds per minutes)) for 30 min, on 100 KD microfilter Amicon. A waste volume of about 1.6 ml containing free (non-conjugated) protein was obtained. An equal volume of buffer was added to the concentrate. The product was subjected to dialysis on 100 KD - 200 KD molecular cut-off tube, depending on the carrier M.W.,  
20 against 0.1 M acetate buffer, at a pH of 5.5. Four dialysis passages in the cold for a total of about 30 h were performed.

c) Conjugation with the hapten

15 to 50 mol of hapten per mol of poly-amino acidic copolymer having a MW of 50 KD was used.

25 A solution of oxidized protein/poly-(D-Glu,D-Lys) copolymer conjugate (having a MW of 50 KD), in a poly-(D-Glu,D-Lys) copolymer concentration of 8 to 10 µM/ml (micromoles/milliliter) in acetate

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buffer 0.1M pH 5.5, and hapten solutions, in concentrations of 30 to 100 µM/ml, in acetate buffer 0.1M pH 5.5 and optionally solvents to solubilize, were prepared. The following ingredients were mixed:

- a solution of oxidized protein/poly-(D-Glu,D-Lys)

- |   |                                       |        |
|---|---------------------------------------|--------|
| 5 | copolymer conjugate in acetate buffer | 1.0 ml |
|   | - 0.1 M acetate buffer, pH 5.5        | 1.5 ml |
|   | - hapten solution in acetate buffer   | 1.0 ml |

The solution was added with 65 mg of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (10 mg/10 µl in water CDI solution  
10 to be prepared immediately prior to use). The reaction was continued for 30 min at 37°C. The resulting product was dialyzed against PBS buffer (physiological solution-phosphate buffer) at a pH of 7.4 on a 30 KD molecular cut-off tube. The dialyzate was kept in a refrigerator.

- 15 Method D - Conjugation of hapten with primary -NH<sub>2</sub> groups with a periodate-oxidized proteic substance

Reagents: PBS; 1M carbonate/bicarbonate buffer, pH 9.5; 0.1 M sodium periodate (21 mg/ml); ethylene glycol (1 ml ethylene glycol in 17 ml distilled water); sodium boron hydride (4 mg/ml distilled water  
20 solution, prepared immediately prior to use).

Procedure: 2 mg of proteic substance in 0.5 ml of 0.1 M sodium periodate was dissolved in 0.1 M acetate buffer, pH 5.5. The solution was stirred at room temperature for 20 min, added with 30 µl of 1 M glycerol to block the action of excess periodate, stirred for  
25 additional 5 min, filtered through fine Sephadex G 25 column (1x5, equilibrated with 0.1 M carbonate/bicarbonate buffer, pH 9.5).

A brown fraction was collected, which provides a O.D. read in UV at

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280 nm. 10 mg of hapten in little saline physiological solution (about 1 ml) and then 0.1 ml of 1 M carbonate/bicarbonate buffer were added. After 2-h stirring at room temperature, 0.2 ml of 4 mg/ml sodium boron hydride was added. The resulting solution was allowed to stand for 2 5 h, then dialyzed against PBS overnight, centrifuged for 15 min (10,000 rpm), and added with an equivalent volume of glycerol.

**Method E - Conjugation of hapten with protein in the presence of glutaraldehyde**

10 mg of proteic substance was dissolved in 1 ml of 0.1 M phosphate 10 buffer at a pH of 6.8, wherein the hapten, in a proteic substance/hapten molar ratio equal to 1:15, had been dissolved. The solution was added with 50  $\mu$ l of 1% glutaraldehyde in 0.1 M phosphate buffer at a pH of 6.8, caused to react at room temperature for 3 h, added with 50  $\mu$ l of lysine 1M pH<sub>7</sub> (pH 6.6-7.4) in PBS to block the 15 glutaraldehyde reactive groups. The resulting product was allowed to stand at room temperature for 1-2 h or overnight in a refrigerator, and then dialyzed against PBS buffer on a 10 KD molecular cut-off tube, overnight, centrifuged for 20 min (10,000 rpm), added with an equivalent volume of glycerol, and kept at a temperature ranging from 20 4°C to -20°C.

**Method C - Conjugation of periodate-oxidized hapten with proteic substance**

2 mg of hapten with glucydic -OH groups were dissolved in 0.5 ml of 0.1 M NaIO<sub>4</sub> dissolved in 0.1 M acetate buffer, pH 5.5. The solution 25 was allowed to stir at room temperature for 20 min, added with 30  $\mu$ l of 1 M glycerol, stirred for additional 30 min, cooled to 4°C for about 1 h and a half, added with NaOH to a pH of about 9.0 and with



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0.1 M carbonate/bicarbonate buffer until obtaining a final volume of 1 ml, containing 8.0 mg of proteic substance. The resulting solution was stirred at room temperature for 2 h, added with 200 µl of 4 mg/ml sodium boron hydride, allowed to stir at room temperature for 2 h, and  
5 dialyzed against physiological solution.

Method F -Conjugation with a proteic carrier through disulfide bonds  
(SPDP method)

Reaction Scheme for haptens with -SH groups (e.g. propylthiouracil):

A solution of 1 mg/ml of BSA (M.W. 65,000 D) in PBS buffer (0.1M, pH  
10 7.4), cooled with ice, is added under magnetical stirring with 5 µl of  
a 10 mM ethanolic solution of N-succinimyl-3-(2-pyridylthio)-  
propionate (SPDP). The reaction mixture is reacted at room temperature  
(e.g. 23°C) for 30-60 minutes, e.g. 30 minutes, under stirring at a  
low rate, avoiding foam formation. At the end of reaction, the  
15 solution is immediately filtered through a sephadex column 25 (1x10  
cm) with phosphate buffer 0.1M, pH 7.4.

The fractional containing the 2-pyridyldisulfide derivative are collected as 1 ml fractions, and stored at 4°C under nitrogen, or used immediately afterwards.

20 Hapten is reacted in a molar ratio of from 15:1 to 50:1, e.g. 20:1,  
with respect to BSA, 1 ml of solution containing the BSA-S-S-2-pyridyl  
derivative is added with 2 ml of a hapten solution in PBS, in the  
aforementioned molar ratios, and reacted at room temperature for 30-120  
minutes, e.g. 60 minutes, affording the conjugate product in a 70%  
25 yield. The conjugate can be directly used as specific immunogen for  
production of antibodies, without any purification.

PREPARATION OF IMMUNOGENS

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Example 1A - Immunogen derivative of haptens conjugated with a proteic carrier represented by the oxidized gamma globulin/poly-(D-Glu,D-Lys) copolymer conjugate

Method B was repeated using gamma-globulin IgG having a MW of 160,000 as a protein, each time using one of the following haptens: neomycin, gentamicin, streptomycin, amphotericin, cycloserine, amphetamine, sulphamethoxazole. Example 1B - Immunogen of tetracyclines

Oxytetracycline and doxycycline were derivatized by treatment with diazotized para-amino benzoic acid, then conjugated with bovine serum albumin (BSA having a MW of 65,000) in the presence of N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide according to the process described by E.C. Beuvery, G.J.A. Speijers, B.I.G. Lutz, D. Freudenthal, V. Kanhai, B. Haagmans, and H.J.G.M. Derks, in Develop. Biol. Standard, S. Karger, Basel (1986), 63, 117.

Example 2 - Immunogen of amino glycosidic antibiotics

Method C was repeated using BSA having a MW of 65,000 as a protein, and each time using as a hapten one of the following compounds: neomycin B, gentamicin, streptomycin, amphotericin and idoxuridine.

Example 3 - Immunogens of BSA-conjugated drugs in the presence of glutaraldehyde

Method E was repeated using BSA having a MW of 65,000 as a proteic substance, and each time using as a hapten one of the following compounds: neomycin, gentamicin, streptomycin, amphotericin; cycloserine; amphetamine, sulphamethoxazole.

Example 4 - Immunogen of cephalosporins

Ceftazidime, cefotaxime, cephalixin were treated with bovine serum albumin (BSA, having a MW of 65,000), in the presence of a

carbodiimide as a conjugation reagent, operating under the conditions described by the reference of E.C. Beuvery, reported in Example 1B.

Example 5 - Immunogens of penicillins 6-aminopenicillanic acid and penicilloic acid were treated with bovine serum albumin, according to  
5 the direct carbodiimide conjugation method. Example 6 - Immunogen of sulphonamides

Sulphamethoxazole [3-sulphanylamido-5-methylisoxazole] was conjugated with gamma-globulin/copolymer in the presence of a carbodiimide, according to method B. Example 7 - Immunogen of anti-thyroid 2-  
10 thiouracyl derivatives

Propylthiouracyl was conjugated with BSA in the presence of SPDP, under the operating conditions above described. Example 8 - Immunogens of diethylstilbestrol [3,4-bis-(p-hydroxyphenyl)-3-hexene], 17- $\beta$ -estradiol, zeranol [lactone of 6-(6,10-dihydroxyundecyl) $\beta$ -resorcinic  
15 acid], trenbolone [17- $\beta$ -hydroxy-estra-4,9,11-trien-3-one]

Said immunogens were obtained conjugated the drugs of the title, previously derivatized as above described in the present text, with BSA, in the presence of a carbodiimide, according to method A.

Example 9 - Immunogen of olaquinox (OLA)

20 - 9a - Preparation of diazotized PABA

p-Aminobenzoic acid (PABA) (8.2 mg;  $6 \times 10^{-4}$  moles) was dissolved in 1N HCl (6 ml). The solution was ice cooled and added with NaBr (10 mg). A  $\text{NaNO}_2$  (37 mg;  $5.4 \times 10^{-4}$  moles) solution in  $\text{H}_2\text{O}$  (2ml) was  
extemporarily prepared, ice cooled and added dropwise under stirring  
25 in 10 minutes to the PABA solution. Stirring was continued for one additional hour at 4°C.

- 9b- Preparation of olaquinox-PABA azoderivative

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OLA (142 mg;  $5.4 \times 10^{-4}$  mole) was dissolved in 2N NaOH<sub>4</sub> (about 6 ml), adjusting the volume to 10 ml with water. This solution was ice cooled, then added with diazotized PABA at small portions, smaller than drops. During the diazotized PABA addition pH was adjusted to 9-  
5 10 by 2N NaOH addition. At the end of diazotized PABA addition, pH is adjusted to 9 and the solution is stirred in ice for additional 4 hours.

- 9c - Preparation of OLA-OVA conjugate (control)

Conjugation was carried out by reacting chicken ovoalbumin (OVA) (4 ml  
10 of a 10mg/ml solution = 40 mg) with the diazo-OLA derivative solution (8 ml) (pH= 6.0) in the presence of carbodiimide, for 15 hours at the laboratory temperature (at about + 10°C). At the end of reaction, the excess of carbodiimide was removed by dialysis against saline solution.

15 Molar ratios OVA-OLA :

8 ml azo-OLA :  $2.16 \times 10^{-4}$  mole azo-OLA

4 ml OVA : 40 mg :  $1 \times 10^{-6}$  mole OVA

Molar ratio diazo-OLA:OVA = 200 :1

- 9d - Preparation of OLA-HSA conjugate

20 The reaction was carried out under the same conditions used for the OLA-OVA conjugate, by using HSA (4 ml solution containing 40 mg;  $6 \times 10^{-7}$  mole of HSA), azo-OLA derivative solution (4ml); carbodiimide 100 mg.

Molar ratios HSA-OLA (Immunogen):

25 4 ml azo-OLA :  $1.08 \times 10^{-4}$  mole azo-OLA

4 ml HSA : 40 mg :  $6 \times 10^{-7}$  HSA

Molar ratio diazo-OLA:HSA = 166 :1

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The OLA-HSA conjugate was injected in animals to produce antibodies as described below, affording IgG in yields of 25.3 mg/ml.

The OLA-OVA conjugate was used as control in the ELISA test hereinafter described to test the antibody specificity: an antibody  
5 specific for a given hapten gives a response both with the injected immunogen and with a conjugate of the same hapten to a different proteic carrier.

**Example 10 - Immunogen of oxytetracycline (OTC)**

OTC 1 mg =  $2.17 \times 10^{-6}$  moles

10 In this and in the above Examples the carbodiimide was N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide.

**- 10a - Preparation of diazotized PABA**

Diazotized PABA was prepared according to Example 9a using PABA (41 mg), 1N HCl (3 ml), NaBr (5 mg), NaNO<sub>2</sub> (18.5 mg).

15 Final volume : 4.9 ml.

**- 10b - Preparation of OTC-PABA azoderivative**

OTC (100 mg) was dissolved in 2N NaOH (2 ml), adjusting the volume to 5 ml with water. The OTC solution ( $2.17 \times 10^{-4}$  moles) was added with the diazotized PABA solution (3.2 ml;  $1.96 \times 10^{-4}$  moles) and treated  
20 as previously described for Example 9b.

Final volume : 8.2 ml.

**- 10c - Preparation of OTC/OVA conjugate (control)**

OTC was conjugated with OVA under the same reaction conditions of Example 9c.

25 The following amounts of OVA and OTC were used:

OVA : 4 ml of a solution containing 40 mg ( $1 \times 10^{-6}$  moles)

azo-OTC : 2 ml containing  $5.28 \times 10^{-5}$  mole

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carbodiimide : 100 mg

Molar ratio OTC : OVA = 52.8 : 1 = about 53

- 10d - Preparation of OTC-HSA conjugate

OTC was conjugated with HSA under the same reaction conditions of

5 Example 9d.

The following amounts of HSA and OTC were used:

HSA : 4 ml of a solution containing  $6 \times 10^{-7}$  moles

azo-OTC : 3 ml containing  $7.92 \times 10^{-5}$  mole

carbodiimide : 100 mg

10 Molar ratio OTC : HSA = 792 : 6 = 132

After immunization of animals by injecting the OTC.HSA conjugate, IgG were obtained in a yield of 27.07 mg/ml.

As above reported, the OTC/OVA conjugate was used in the ELISA test as control.

15 Example 11 - Immunogen of sulfamethazine (SM)

- 11 a - Preparation of diazotized SM

SM : 1 mg ( $3.59 \times 10^{-6}$  moles)

SM (25 mg;  $0.9 \times 10^{-4}$  mole) was dissolved in conc. HCl (2 ml) + H<sub>2</sub>O (2 ml), the resulting solution was ice cooled then added with NaBr (2.5  
20 mg).

The SM solution thus obtained was added dropwise in 10 minutes under stirring, under ice cooling, with a solution of NaNO<sub>2</sub> in H<sub>2</sub>O (2 ml). The resulting mixture was stirred for one additional hour, under ice. Then the volume was adjusted to 12 ml.

25 - 11b - Preparation of SM-AZO-OVA conjugate (control)

OVA : 4 ml containing 40 mg;  $1 \times 10^{-6}$  mole

Diazo-SM : 3 ml containing  $7.5 \times 10^{-6}$  mole  $\times 3 = 2.25 \times 10^{-5}$  mole

The solution containing the diazotized-SM was added dropwise, under stirring, in 15 minutes, to the OVA solution, maintaining the pH at about 9.0-10.0 by 2N NaOH addition. At the end of addition, the reaction mixture is kept under stirring for additional 4 hours at 4°C.

- 5 The conjugate solution is dialyzed against saline solution. Molar ratio SM : OVA = 22

- 11c - Preparation of SM-AZO-HSA conjugate

Diazo-SM was conjugated with HSA under the same reaction conditions of Example 11b.

- 10 The following amounts of HSA and Diazo-SM were used:

HSA : 2 ml of a solution containing 20 mg;  $3.0 \times 10^{-7}$  moles

diazo-SM : 4.5 ml containing  $3.37 \times 10^{-5}$  mole

Molar ratio SM : HSA = 112

- After immunization of animals by injecting the SM-conjugate yields of  
15 specific IgG of about 30.2 mg/ml were obtained.

Analogously to what above reported, the SM-OVA conjugate was used as control in the ELISA test.

Example 12 - Production of specific IgG antibodies to immunogens

- A. Production of antibodies. The immunogens prepared as described in  
20 the examples reported above were administered to goats or horses, in the presence of Freund's adjuvant, according to the scheme described by A. Johnstone and R. Thorpe, in *Immunochemistry in Practice*, p. 28, A. Johnstone and R. Thorpe, eds., Blackwell Sci. Publ., Oxford (1982), more particularly following the process described below:

- 25 **Primary immunization:** The animals were given an oil/water emulsion containing the immunogen (0.1-2 mg per kg of body weight) in PBS (2 ml), emulsified with FcA (Freund's complete adjuvant) in the presence

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of an emulsifier, by subdividing the dose into several injections administered intradermally in different parts of the body.

Booster: 14 to 21 days after the primary injection, goats or horses were given a preparation containing the immunogen (0.1-2 mg) in PBS (2 ml) and FIA (Freund's incomplete adjuvant), by intramuscular route.

The immune response was checked as described hereinafter on a blood sample taken from the femoral artery, 20 to 40 days after booster administration.

The serum containing IgGs was withdrawn and used to prepare immunodetoxifiers consisting of the F(ab')<sub>2</sub> fraction of the antibody and to purify the IgGs to be used in the diagnostic test according to the present invention.

B. Purification of IgGs from the serum. Said purification was carried out by chromatography on 1 M DEAE-Trysacryl, eluting with 0.025 M Trys-HCl (0.035 M/NaCl) buffer, having a pH of 8.8. The immunoglobulins of class IgG, being 98-99.5% pure, were eluted, whereas those belonging to the other classes were retained. Chromatography on DEAE-Trysacryl column was conducted on plasma cryoprecipitated to remove, e.g., fibrinogen, chylomicrons, lipoproteins, whereby the chromatographic columns might be damaged, and was preceded by a passage on ULTROGEL Aca 202 column, eluting with the aforesaid buffer, for desalination.

The operating process is described, e.g., in the technical bulletin provided by the supplier: "Reactifs IBF", "Preparation of Albumin and IgGs by Direct Chromatographic Separation of DEAE- and CM-Trysacryl M", by J. Saint-Blancard, J. Fourcart, E. Boschetti, P. Girot.

Once chromatography had been performed, IgGs were concentrated again



to the original volume on 10 KD molecular cut-off microfilter Amicon and dialyzed for 24 h against physiological saline solution.

Titration of the proteic content was carried out by the biuret method.

The antibody activity was evaluated according to ELISA, an  
5 immunoenzymatic method of competitive type, according to which the  
hapten is bound to the inert support, and the antibody concentration  
is detected by means of a anti-antibody-enzyme (HRP) conjugate or to  
the precipitin method or other conventional methods.

C. Proportioning of IgGs in the serum of the IgG-producing animal:

10 IgGs were determined quantitatively by conventional methods, e.g., by  
passive hemoagglutination against the hapten conjugated covalently  
with sheep erythrocytes, (E.H. Relyveld, P. Saliou and N.Le Camp,  
L'eurobiologiste, 1990,tome XXIV, No.187, Pag. 219-228) titrating the  
IgG antibody present in the sample, if any, in wells added with  
15 erythrocytes sensitized through linking with the hapten. An opaque and  
homogeneous well surface indicated the presence of antibodies in the  
assayed sample.

IgGs dosage on microplate with "V" wells, on serum, plasma or whole  
blood, as follows:

- 20 1. 1:20 dilution in PBS of a sample (animal serum containing the  
IgGs);
2. Buffer distribution (50 µl) into wells;
3. Addition to wells of diluted samples (50 µl)
4. Addition to well of 50 µl sensitized erythrocytes;
- 25 5. 1 minute stirring
6. Results readout: positive ones = homogeneous opaque well surface;  
negative ones = big dense precipitate at the well bottom; weakly

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positive = small precipitate at the well bottom.

Samples can be quali-quantitatively evaluated, comparing samples with standard reference preparations serially diluted to known concentration.

5 Example 13 - Preparation of F(ab')<sub>2</sub> fragments

For 1000 ml serum. The serum pH was adjusted to about 7 (e.g. 7.4). The serum was slowly added at room temperature with 1000 ml of 32% PEG<sub>6000</sub> in water, having a pH of 7 (final PEG 16%) and precipitated for at least 30 min at room temperature. Precipitate PI was recovered  
10 by filtration through Laurent's filter No. 1. The filtrate was discarded. Precipitate PI was dissolved in 1,500 ml of 0.9% NaCl (3/4 of 2,000 ml, i.e., 3/4 of the volume of the first precipitation). The pH was adjusted to 7. The solution was added slowly with 1,500 ml (v/v) of 28% PEG<sub>6000</sub> solution [final PEG 14%] and precipitated for at  
15 least 30 min at room temperature. Precipitate PII was recovered by filtration. The filtrate was removed. Precipitate PII was dissolved in 1,000 ml of 0.9% NaCl and added with 16.5 g of 0.22 M glycine. After adjusting the pH to 3 with 1N HCl, the solution was placed on water bath at 31.5°C.

20 When the temperature reached the desired value, the solution was added with 200,000 U pepsin (200 U/ml PII), left on water bath at 31.5°C for 90 min, and stirred at 15-min intervals. The pepsin dregs were eliminated by 10-15 minutes' centrifugation (3,000 rpm).

The supernatant concentration was adjusted to 0.5 M NaCl from the  
25 initial concentration of 0.15 M, by addition of 20 g NaCl. The pH was adjusted to 7.5 (pepsin neutralization) and then to 5. The solution was added with 350 ml of 40% PEG<sub>6000</sub> [final PEG 10%], precipitated for

at least 30 min at room temperature. The filtrate, SIId, was recovered. The optical density (OD) was read at 280 nm (an aliquot of the filtrate was diluted 1:20 to evaluate protein concentration). The pH was adjusted to 7 by addition of 1N NaOH. Some time later, 900 ml  
5 (equal volume) of 50% PEG<sub>6000</sub> was added (final PEG = 30%). The resulting solution was precipitated for at least 30 min at room temperature.

The precipitate obtained was recovered, dissolved in the smallest quantity required of 0.9% NaCl (200 ml) and dialyzed against 0.9% NaCl  
10 (2 litres), overnight, at 4°C. The dialyzate was recovered, contrifuged for 15 min (4,000 rpm). The optical density was measured and the concentration of F(ab')<sub>2</sub> was adjusted to 10 mg/ml.

The product obtained was filtered through 0.45 µm and successively 0.2 µm filters and distributed in tubes (20 mg/2ml/tube). A sterility test  
15 (100 µl Fab')<sub>2</sub> + 0.9 ml of medium with serum without antibiotic) was performed.

#### Example 14 - ELISA-type competitive diagnostic assay for the determination of circulating drugs

The method was analogous to that described by A. Voller, D.E. Bidwell,  
20 and A. Bartlett, in Bull. Wld. Health Org., 53, 55 (1976).

A. Adhesion of IgG antibody to the solid phase. A purified IgG solution in 1 M carbonate buffer, pH 9.5, with 50 µg/ml protein content, determined by the biuret method, was prepared. Each well of a polystyrene microplate was added with 100 µl solution and incubated at  
25 37°C for 2 h; then it was added with 100 µl of a 2% (w/v) casein solution in 0.05 M PBS buffer, at pH 7.4, and allowed to saturate overnight in a refrigerator. Casein was removed and the wells were

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washed three times with PBS buffer. The microplate was dried in a desiccator with silica gel, under vacuum.

B. Sample solution: animal serum to be checked.

C. Enzyme-labelled compound to be proportioned: the compounds to be  
5 proportioned (haptens) were linked to horse-peroxidase (HRP) by different methods according to their structure.

Conjugates wherein the drug is labelled with oxidized peroxidase (HRP)/poly-(D-Glu,D-Lys) copolymer conjugate

Method B was repeated using HRP having a MW of 45,000 as the protein.  
10 Conjugation was each time carried out with the haptens specified for immunogens preparation according to Method B in Example 1A.

Conjugates of drug or metabolite containing -NH<sub>2</sub> groups and peroxidase (HRP) in the presence of glutaraldehyde

Method E was repeated using HRP having a MW of 45,000 as a protein  
15 and, as haptens, the haptens used for immunogens preparation according to method E in Example 3.

Conjugates of aminoglycosidic antibiotics and HRP

Method C was repeated using HRP having a MW of 45,000 as a protein and, as haptens, the haptens used for immunogens preparation according  
20 to method C in Example 2.

Immunodiagnostic assay

Reagents and instrumentation: The diagnostic kit includes:

1. Solid phase: polystyrene 96-well micro titrating plate (12 eight-well rows) incorporating the already adsorbed antibody specific for  
25 the drug to be proportioned.
2. PBS/casein diluting buffer (2 x 50 ml).
3. PBS/Tween concentrated wash solution (1 x 50 ml).

4. Conjugate (enzyme-labelled hapten): peroxidase-labelled haptens (1 x 320 µl).
5. Chromogen: TMB (tetramethylbenzidine) buffered solution (1 x 6 ml).
6. Substrate: H<sub>2</sub>O<sub>2</sub> buffered solution (1 x 6 ml) [enzyme substrate].
- 5 7. Reagent to stop the reaction: 0.5-2N sulphuric acid (4 x 100 µl).
8. Positive controls: 8a. Standard 1; 8b. Standard 2; 8c. Standard 3; 8d. Standard 4 (the standard used is the diluted serum of animal treated with the drug to be proportioned, whose concentration has been determined by another analytical method known for said drug. It is thus possible to determine the various forms of the circulating drug or metabolite, be it free and/or linked to proteins and to prepare a calibration curve of drug or metabolite hematic levels.

In some cases, positive controls may be represented by milk.

9. Negative control: (1 x 100 µl) (serum of untreated animal).
- 15 All reagents and the plate are to be kept at 2 to 8°C, shielded from light. The film covering the rows is to be removed immediately prior to use. Reagents are to be used after heating to room temperature.

Procedure: The dilutions indicated below are to be made immediately prior to use:

- 20 Dilute reagent 4 with reagent 2 (1:40; e.g. from 25 µl to 1 ml for each 8-well row).

Dilute reagent 5 with an equal volume of reagent 6 (e.g. 1 ml + 1 ml for each 8-well row).

Dilute reagent 3 with physiological solution (1:10).

- 25 Samples to be assayed: if the assay is not performed during the day of sample withdrawal, the sample is to be frozen at -20°C.

Serums inactivated at 56°C shall not be used.

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Samples: dilute 1:100 with reagent 2.

Controls 8a, 8b, 8d, 9: dilute 1:100 with reagent 2.

Assays are carried out in duplicate on two well rows, by adding each well with 100 µl of the reagents indicated in Table 1, according to the scheme reported therein:

Table 1

	Row No. 1	Row No. 2
1	W	Pd
2	W	Pd
10 3	Pa	N
4	Pa	N
5	Pb	S
6	Pb	S
7	Pc	-
15 8	Pc	-

W = White (diluting buffer-reagent 2); Pa, Pb, Pc, Pd = Positive controls corresponding to dilute reagents 8a, 8b, 8c, 8d); N = Negative control (dilute reagent 9); S = Sample under examination, in the dilute form.

- 20 Rows are covered with an adhesive film and are incubated at 37°C for 1 h. Wells are washed three times with 350 µl wash solution (reagent 3). Once all wash residues have been carefully eliminated, each well is added with 100 µl of reagents in the following order:
- conjugate (reagent 4)
  - 25 - chromogenic substrate (reagent 5).

After each addition of reagent, the row is covered, incubated at 37°C for 1 h. Before being added with the successive reagent, the wells are

washed as specified above.

The reaction is stopped by addition of reagent 7 (50 µl/well). Mixing is carried out by a plate agitator, the optical density (OD) is read at 450 nm within 30 min, the zero value having been set as White. The  
5 assay is considered valid when the mean value of the negative control is  $E_{(\text{negative})} \leq 0.100$ .

Qualitative determination: The positivity cutoff value ranges from  $0.250 \leq E_{(\text{cutoff})} \leq 0.350$ . The samples giving results below the cutoff value are "negative", whereas the samples giving results above the  
10 cutoff value are "positive". The determination is to be repeated with results ranging within the cutoff value interval.

Quantitative determination: The kit is provided with 4 standard serum dilutions allowing the plotting of a calibration curve. To calculate the hapten concentration (µg/ml) in the sample, it is enough to  
15 extrapolate the OD value of the sample from the calibration curve previously plotted.

By way of example, Fig. 1 shows a calibration curve reporting the drug concentration of standard solutions 1, 2, 3, and 4, expressed as Log µg/ml on abscissa, and the OD on ordinate. The drug concentration that  
20 may be determined in the animal varies from ng to µg.

#### Example 15 - Use of $F(ab')_2$ fragments for the detoxification from drugs

Bovine animals were subjected to treatment with the drugs (whose immunogens were prepared as described above), for instance neomycin,  
25 sulfamethoxazole, propylthiouracil, zeranol, cycloserin, amphetamine, olaquinox, oxytetracycline, sulfamethazine for a time securing a pharmacological effect. The drug or metabolite concentration in the

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serum was determined by the aforementioned competitive ELISA assay, calculating the drug concentration in the serum by the calibration curve obtained as reported above for ELISA assay.

Before detoxification, 100% positive values were observed (OD above  
5 the cutoff value ranging from 0.250 to 0.350; cf. Fig. 1). The drug concentration in the serum was determined on the basis of the calibration curve. The animals to be treated were then administered, by the i.m. route, 1 mol of  $F(ab')_2$  specific for said drug/2 mols of drug or metabolite present in the serum.

10 The determination of the drug concentration in the serum by ELISA assay was repeated 48-72 h from  $F(ab')_2$  administration. The detoxifying treatment was effective when the drug values in the serum were negative, i.g., below the OD cutoff value, as defined above. If positive values were still found, the detoxifying treatment was  
15 repeated by administering 1 mol  $F(ab')_2$ /2 mol drug.

The drug level in the blood was substantially reduced below the aforesaid cutoff value by the aforementioned detoxifying treatment, after a number of administrations generally ranging from 1 to 3.



## Claims

- 1 1. F(ab')<sub>2</sub> fragments of IgG immunoglobulins active as specific  
2 antibodies to a drug or a metabolite thereof selected among:  
3 antibiotic; sulphonamide; antiviral drug; antiparasitic drug;  
4 antimycotic drug; antitubercular drug; sympathicomimetic drug; anti-  
5 thyroid agent; estrogenic, androgenic or progestinic substances;  
6 cortisone and corticosteroid analogs; endogenous hormone; auxinic  
7 drug; amphetamine growth stimulant.
- 1 2. The fragment as claimed in claim 1, wherein the antibiotic is  
2 selected among tetracyclines, amino glycosidic antibiotics,  
3 cephalosporins and penicillins; the sympathicomimetic drug is a  $\beta$ -  
4 agonist having 2-phenylethylamine structure; the anti-thyroid agent is  
5 a 2-thiouracyl derivative; the sulphonamide is substituted at the  
6 sulphonamide nitrogen with an isoxazole ring; growth stimulant has a  
7 quinoline structure.
- 1 3. The fragment as claimed in claim 1, wherein the antibiotic is  
2 selected out of oxytetracycline, doxycycline, neomicyn B, gentamicin  
3 C, tobramycin, kanamycin, amikacin, and streptomycin, ceftazidime,  
4 cefotaxime, cephalixin, a penicillin; the sulphonamide is  
5 sulphamethoxazole or sulphamethoxazine; the antiviral drug is  
6 idoxuridine; the antimycotic drug is amphotericin or ketoconazole; the  
7 antitubercular drug is cycloserine or isoniazid; the sympathicomimetic  
8 drug is clenbuterol or cimaterol; the anti-thyroid agent is  
9 methylthiouracil or propylthiouracil; the estrogenic substance is  
10 diethylstilbestrol, 17- $\beta$ -estradiol or zeranolol; the androgenic  
11 substance is trenbolone or 19-nortestosterone; the progestinic  
12 substance is progesterone; the corticosteroid analog substance is

13 dexamethasone; the endogenous hormone is somatotrophin; the auxinic  
14 drug is selected out of virginiamycin, zincobacitracin, avoparcin; the  
15 amphetaminic drug is amphetamine or methamphetamine. the growth  
16 stimulant is olaquinox.

1 4. Process for the preparation of  $F(ab')_2$  fragment as claimed in any  
2 of claims 1 to 3, comprising removing by digestion with pepsin the Fc  
3 fragment of the corresponding IgGs, obtained from the serum of animals  
4 treated with an immunogen derivative obtained by conjugation of a  
5 hapten selected among the drug or metabolite to be eliminated from  
6 organism, or structurally related derivatives thereof, with a proteic  
7 or polysaccharide carrier.

1 5. A process as claimed in claim 4, wherein the hapten used for the  
2 preparation of the immunogen is the drug, the metabolite or derivative  
3 thereof functionalized with functional groups suitable for covalent  
4 conjugation with the carrier.

1 6. A process as claimed in claim 5, wherein the immunogen derivative  
2 is selected among the following hapten/proteic carrier conjugates:

3 a) a product obtained by conjugation of a hapten containing primary  
4 amino  $-NH_2$  groups or  $-COOH$  groups, with a proteic carrier in the  
5 presence of a carbodiimide;

6 b) a product obtained by conjugation, in the presence of a  
7 carbodiimide, of a hapten containing primary amino  $-NH_2$  groups, with  
8 an oxidized proteic carrier/synthetic poly-amino acid copolymer  
9 conjugate, wherein the carrier has been previously oxidized with an  
10 oxidizing agent of the  $-C-OH$  groups to  $C=O$  groups then treated with a  
11 reducing agent;

12 c) a product obtained by conjugation of a hapten containing  $-C-OH$

13 groups, previously oxidized with an oxidizing agent of the -C-OH  
14 groups to C=O groups, with a proteic carrier, followed by reduction  
15 with NaBH<sub>4</sub>;

16 d) a product obtained by conjugation of a hapten containing primary  
17 amino -NH<sub>2</sub> groups, with a proteic carrier previously oxidized by  
18 treatment with an oxidizing agent of the -C-OH groups to C=O groups,  
19 followed by reduction with NaBH<sub>4</sub>;

20 e) a product obtained by conjugation of a hapten containing primary  
21 amino -NH<sub>2</sub> groups with a proteic carrier, in the presence of  
22 glutaraldehyde;

23 f) a product obtained by conjugation of a hapten containing one or  
24 more thiol groups with a proteic carrier previously functionalized  
25 with a disulfide reagent;

26 g) a product obtained by conjugation of a hapten containing one or  
27 more aromatic groups reactive towards diazonium salts, with a proteic  
28 carrier, in the presence of a bis-diazo reagent;

29 h) a product obtained by conjugation of a diazotized hapten with a  
30 proteic carrier.

1 7. A process as claimed in claim 6, wherein the proteic carrier has a  
2 molecular weight of 40,000 to 450,000 Daltons, and in case b), the  
3 poly-amino acid copolymer has a molecular weight of 20,000 to 100,000  
4 Daltons;

1 8. A process as claimed in claim 6, wherein for conjugates a), c), d),  
2 e), f) or g), the proteic carrier is bovine serum albumine (BSA) or  
3 HSA human serum albumine, and for conjugate b) the proteic carrier is  
4 selected among gamma-globulin IgG and a polysaccharide with molecular  
5 weight of 50,000-200,000 Daltons.

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- 1 9. The process as claimed in claim 6, wherein for conjugate product a)  
2 the hapten is selected among :
- 3 - a drug or metabolite previously functionalized so to introduce at  
4 least one -COOH group, selected among:
- 5 i) drugs or metabolites with aromatic groups activated towards  
6 diazotization, previously functionalized by treatment with diazotized  
7 para-amino benzoic acid; ii) drugs or metabolites with one or more  
8 keto groups, previously reacted with o-(carboxymethyl)-hydroxylamine  
9 to give the corresponding o-(carboxymethyl)-oxyme;
- 10 iii) drugs or metabolites with one or more keto groups, previously  
11 reacted with para-hydrazino benzoic acid to give the corresponding  
12 hydrazones containing a -COOH group;
- 13 - a drug or metabolite with at least one secondary amino groups,  
14 previously converted into primary amine, by reacting it with N-(4-  
15 bromobutyl)phthalimide, then hydrolyzing the phthalimido group.
- 1 10. The process according to claim 6, wherein the immunogen derivative  
2 is selected among:
- 3 - conjugate a), wherein the hapten is selected among cephalosporins,  
4 6-amino penicillanic acid, penicilloic acid, and haptens previously  
5 functionalized selected among:
- 6 i) drugs or metabolites with phenolic groups, previously  
7 functionalized by treatment with diazotized para-amino benzoic acid,  
8 selected among tetracyclines, steroids with one or more phenolic  
9 groups, 17-beta-estradiol, diethylstilbestrol, zeranol and compounds  
10 with a quinoxaline ring;
- 11 ii) ketokonazole, previously reacted with o-(carboxymethyl)-  
12 hydroxylamine to give the corresponding o-(carboxymethyl)-oxyme;

- 13 iii) trenbolone, previously reacted with para-hydrazino benzoic acid;  
14 and
- 15 iv) metamphetamine, previously reacted with N-(4-  
16 bromobutyl)phthalimide, followed by removal of the phthalimido group to  
17 give the corresponding primary amine;
- 18 for conjugate a) the proteic carrier is bovine serum albumin, and the  
19 carbodiimide is N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide;
- 20 - conjugate b), wherein the hapten is selected among glycosidic  
21 antibiotics or antimycotic drugs, cycloserine, amphetamine,  
22 sulphonylamides, cimaterol, clenbuterol; the enzyme is gamma globulin  
23 IgG oxidized with periodate and conjugated with poly-(D-Glu,D-Lys)  
24 then treated with a reducing agent;
- 25 - conjugate c), in which the oxidized hapten is selected among amino  
26 glycosidic antibiotic or antimycotic drug, and idoxuridine, and the  
27 proteic carrier is bovine or human serum albumin;
- 28 - conjugate d), in which the hapten is an amino glycosidic antibiotic  
29 and the proteic carrier is periodate-oxidized bovine or human serum  
30 albumin;
- 31 - conjugate e), in which the hapten is selected among amino glycosidic  
32 antibiotic or antimycotic drug, amphetamine, sulphonylamides,  
33 cycloserine, cimaterol and clenbuterol, and the proteic carrier is  
34 bovine or human serum albumin;
- 35 - conjugate f), in which the hapten is a 2-thiouracil, the proteic  
36 carrier is bovine or human serum albumin, and the disulfide reagent is  
37 N-succinimyl-3-(2-pyridyldithio)propionate (SPDP);
- 38 - conjugate g), in which the hapten is isoniazide, the carrier is  
39 bovine or human serum albumin, and the bis-diazo reagent is bis-

40 diazotized benzidine.

41 - conjugate h), wherein the hapten is a diazotized sulphonamide and  
42 the carrier is human serum albumin.

1 11. The process as claimed in claim 6, wherein the immunogen is  
2 selected among : i) the conjugated between olaquinox reacted with  
3 diazotized p-aminobenzoic acid and human serum albumin in the  
4 presence of carbodiimide; ii) the conjugate between oxytetracyclin  
5 reacted with diazotized p-aminobenzoic acid and human serum albumin in  
6 the presence of carbodiimide; iii) the conjugated between diazotized  
7 sulphomethazine and human serum albumin.

1 12. A process as claimed in claim 6, wherein:  
2 in case b), the oxidized glyco-protein copolymer molar ratio ranges  
3 from 1:5 to 1:30, and the hapten:conjugated copolymer/oxidized protein  
4 molar ratio ranges from 15:1 to 50:1;  
5 in case e) the hapten:proteic carrier molar ratio ranges from 10:1 to  
6 50:1, and the amount of glutaraldehyde ranges from  $10^{-4}$ - $10^{-5}$  Moles  
7 each  $10^{-6}$ - $10^{-5}$  mole of  $\text{NH}_2$  groups of the proteic carrier.

1 13. A process as claimed in claim 5, wherein said immunogen derivative  
2 is administered to the animals selected for IgG immunoglobulins  
3 production by a primary injection and a booster; the serum is  
4 withdrawn and the proteic fraction corresponding to IgG  
5 immunoglobulins is isolated and purified.

1 14. A process as claimed in claim 13, wherein the animals selected for  
2 IgGs production are goats or horses; the immunogen is combined with  
3 Freund's complete adjuvant in the primary injection and with Freund's  
4 incomplete adjuvant in the booster; the primary injection is  
5 administered by the subcutaneous route and the booster by the

6 intramuscular route.

1 15. A process as claimed in claim 5, wherein digestion with pepsin is  
2 conducted on proteic fractions precipitated by addition of  
3 polyethylene glycol.

1 16.  $F(ab')_2$  fragments of IgG immunoglobulins active as specific  
2 antibodies to drugs and metabolites thereof, obtainable according to  
3 the process claimed in any of claims 4 to 15.

1 17. Pharmaceutical compositions including at least a  $F(ab')_2$  fragment  
2 as claimed in any of claims 1 to 4 or 16, combined with one or more  
3 pharmaceutically acceptable excipients and/or diluents.

1 18. Pharmaceutical compositions as claimed in claim 17 in the form of  
2 injectable preparations.

1 19. Use of  $F(ab')_2$  fragments as claimed in any of claims 1 to 4 or 16,  
2 for the preparation of a pharmaceutical composition useful in the  
3 detoxifying treatment of drug-treated humans and animals from said  
4 drug.

1 20. The use as claimed in claim 19, wherein the composition is useful  
2 in the treatment of drug poisoning, overdose or hypersensitivity in  
3 humans or animals.

1 21. The use as claimed in claim 20, wherein the composition is useful  
2 in the treatment, in the human field, of states selected among  
3 allergic manifestations or anaphylactic shock in subjects treated with  
4 anti-inflammatory drugs or antibiotics, and in case of abuse of  
5 pharmacologically active substances.

1 22. The use as claimed in claim 19, wherein the composition is useful  
2 in the detoxification of drug-treated livestock before slaughtering or  
3 milk collection.

1 23. The use as claimed in any of claims 19 to 22, wherein said  
2 fragments are to be administered by the parenteral route.

1 24. IgG-type immunoglobulins active as specific antibodies to drugs  
2 and metabolites thereof, obtainable according to the process claimed  
3 in any of claims 5 to 14.

1 25. IgG-type immunoglobulins active as specific antibodies to drugs  
2 and metabolites thereof, wherein said drugs are as defined in any of  
3 claims 1 to 3.

1 26. Use of IgG immunoglobulins as defined in any of claims 24 to 25  
2 for the preparation of a pharmaceutical composition useful in the  
3 detoxifying treatment from drugs of animals belonging to the same  
4 species as those utilized for IgG production.

1 27. Pharmaceutical compositions including at least an IgG as defined  
2 in any of claims 24 or 25, combined with one or more pharmaceutically  
3 acceptable excipients and/or diluents.

1 28. Pharmaceutical compositions as claimed in claim 27 in the form of  
2 preparations for injection.

1 29. The use of IgG immunoglobulins as defined in any of claims 24 to  
2 26, as diagnostic reagents useful for a competitive ELISA-type assay  
3 for the determination of said drugs or metabolites thereof.

1 30. Competitive ELISA-type diagnostic method for the determination of  
2 the concentration of a drug or metabolite thereof in a biological  
3 fluid sample, comprising the following steps:

4 a sample of biological fluid containing the compound to be assayed is  
5 added to an inert support, sensitized by fixation to IgG  
6 immunoglobulins as defined in claim 24 or 25 active as antibodies to  
7 the compound to be dosed; a conjugate consisting of the enzyme-



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8 labelled compound to be proportioned is added, either in admixture  
9 with the sample to be proportioned or a short time later, after sample  
10 incubation; the mixture is caused to react, is washed and added with a  
11 chromogen capable of reacting with the enzyme; upon colouring, the  
12 readout of the solution optical density is performed, and the quantity  
13 of compound to be proportioned present in the assayed biological fluid  
14 is calculated by difference.

1 31. A process as claimed in claim 30, wherein the inert support is a  
2 plastic plate; the biological fluid is serum, urine or milk; the  
3 enzyme is peroxidase; the chromogenic substrate is ortho-  
4 phenylenediamine or tetramethylbenzidine.

1 32. Diagnostic kit for the determination of a drug and/or metabolite  
2 in the blood, comprising a solid phase incorporating an IgG  
3 immunoglobulin as defined in claim 24 or 25 active as antibody to the  
4 drug and/or metabolite to be determined, a conjugate represented by  
5 the enzyme-labelled compound to be proportioned, and a chromogenic  
6 substrate.

1 33. Conjugate represented by a drug as defined in claims 1 to 3 or  
2 metabolite thereof linked to an enzyme, selected from the group  
3 consisting of:

4 a') a product obtained by conjugation of a hapten containing primary  
5 amino  $-NH_2$  groups or  $-COOH$  groups, with an enzyme in the presence of a  
6 carbodiimide;

7 b') a product obtained by conjugation, in the presence of a  
8 carbodiimide, of a hapten containing primary amino  $-NH_2$  groups, with  
9 an oxidized enzyme/synthetic poly-amino acid copolymer conjugate,  
10 wherein the carrier has been previously oxidized with an oxidizing

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- 11 agent of the -C-OH groups to C=O groups;
- 12 c') a product obtained by conjugation of an enzyme with a hapten  
13 containing -C-OH groups, previously oxidized with an oxidizing agent  
14 of the -C-OH groups to C=O groups, conjugation being followed by  
15 reduction with  $\text{NaBH}_4$ ;
- 16 d') a product obtained by conjugation of a hapten containing primary  
17 amino  $-\text{NH}_2$  groups, with an enzyme previously oxidized by treatment  
18 with an oxidizing agent of the -C-OH groups to C=O groups, conjugation  
19 being followed by reduction with  $\text{NaBH}_4$ ;
- 20 e') a product obtained by conjugation of a hapten containing primary  
21 amino  $-\text{NH}_2$  groups with an enzyme, in the presence of glutaraldehyde;
- 22 f') a product obtained by conjugation of a hapten containing one or  
23 more thiol groups with an enzyme previously functionalized with a  
24 disulfide reagent;
- 25 g') a product obtained by conjugation of a hapten containing one or  
26 more aromatic groups reactive towards diazonium salts, with an enzyme,  
27 in the presence of a bis-diazo reagent,
- 28 h') a product obtained by reaction of a diazotized hapten with an  
29 enzyme.
- 1 34. The conjugate as claimed in claim 33, wherein the hapten is  
2 selected among :
- 3 - a drug or metabolite previously functionalized with at least one -  
4 COOH group, selected among:
- 5 i) drugs or metabolites with an aromatic ring activated towards  
6 diazotation, previously functionalized by treatment with diazotized  
7 para-amino benzoic acid;
- 8 ii) drugs or metabolites with one or more keto groups, previously

9 reacted with o-(carboxymethyl)-hydroxylamine to give the corresponding  
10 o-(carboxymethyl)-oxyme;

11 iii) drugs or metabolites with one or more keto groups, previously  
12 reacted with para-hydrazino benzoic acid to give the corresponding  
13 hydrazones containing a -COOH group; and

14 - a drug or metabolite with at least one secondary amino groups,  
15 previously converted into primary amine, by reacting it with N-(4-  
16 bromobutyl)phthalimide, then hydrolizing the phtalymido group.

1 35. The conjugate as claimed in claim 33 or 34, selected from the  
2 group consisting of:

3 - product a'), wherein the enzyme is horse radish peroxidase, the  
4 carbodiimide is N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide, and  
5 the hapten is selected among cephalosporins, 6-amino penicillanic  
6 acid, penicilloic acid, and haptens previously functionalized selected  
7 among:

8 i) drugs or metabolites with phenolic groups, previously  
9 functionalized by treatment with diazotized para-amino benzoic acid,  
10 selected among tetracyclines, steroids with one or more phenolic  
11 groups, 17-beta-estradiol, diethylstilbestrol, zeranol and drug with a  
12 quinoxaline ring;

13 ii) ketokonazole, previously reacted with o-(carboxymethyl)-  
14 hydroxylamine to give the corresponding o-(carboxymethyl)-oxyme;

15 iii) trenbolone, previoulsy reacted with orto-hydrazino benzoic acid;  
16 and

17 iv) metamphetamine, previously reacted with N-(4-  
18 bromobutyl)phthalimide, followed by removal of the phtalymido group to  
19 give the corresponding primary amine;

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20 - product b'), wherein the hapten is selected among glycosidic  
21 antibiotics or antimycotic drugs, cycloserine, amphetamine,  
22 sulphonylamides, cimaterol and clenbuterol; the enzyme is horse  
23 peroxidase radish oxidized with periodate and conjugated with poly-(D-  
24 Glu,D-Lys), and the carbodiimide is N-ethyl-N'-(3-  
25 dimethylaminopropyl)-carbodiimide;

26 - product c'), in which the oxidized hapten is selected among amino  
27 glycosidic antibiotic, amphotericin, and idoxuridine, and the enzyme  
28 is horse radish peroxidase;

29 - product d'), in which the hapten is an amino glycosidic antibiotic  
30 and the enzyme is oxidized horse radish peroxidase;

31 - product e'), in which the hapten is selected among amino glycosidic  
32 antibiotic, amphotericin, amphetamine, sulphonylamides, cycloserine,  
33 cimaterol and clenbuterol, and the enzyme is horse radish peroxidase;

34 - product f'), in which the hapten is a 2-thiouracil, the enzyme is  
35 horse radish peroxidase and the disulfide reagent is N-succinimyl-3-  
36 (2-pyridyldithio)propionate (SPDP);

37 - product g'), in which the hapten is isoniazide, the enzyme is horse  
38 radish peroxidase, and the bis-diazo reagent is bis-diazotized  
39 benzidine.

40 - product h'), wherein the hapten is a diazotized sulphonamide, and  
41 the enzyme is horse radish peroxidase.

1 36. Process for the preparation of a conjugate of a hapten with a  
2 proteic substance, including:

3 a) treatment of the proteic substance with an oxidizing agent of the  
4 -OH groups to C=O groups;

5 b) treatment of the resulting oxidized proteic substance with a

6 synthetic poly-amino acidic copolymer, followed by reduction with  
7  $\text{NaBH}_4$ ;

8 c) treatment of the resulting oxidized proteic substance/amino acidic  
9 copolymer with the hapten, in the presence of a carbodiimide.

1 37. A process as claimed in claim 36, wherein the oxidation is carried  
2 out with an inorganic periodate, in water, at a pH ranging from 5.5 to  
3 7.5, at a temperature ranging from  $+4^\circ\text{C}$  to  $40^\circ\text{C}$ , shielded from light;  
4 the oxidizing agent is in stoichiometric excess with respect to the  $-\text{OH}$   
5 groups of the proteic substance and, at the reaction end, said excess  
6 is eliminated by addition of a molar excess alcohol, at the oxidation  
7 temperature;

8 in phase b), the copolymer is a copolymer between acid  $\alpha$ -amino acids  
9 and basic  $\alpha$ -amino acids; the reaction is carried out in an aqueous  
10 medium, at an alkaline pH, at a temperature ranging from  $4^\circ\text{C}$  to  $40^\circ\text{C}$ ,  
11 shielded from light; once the reaction is complete, treatment with a  
12 reducing agent is performed;

13 phase c) is carried out in an aqueous medium, at a pH ranging from 5.0  
14 to 6.0, at a temperature ranging from  $30^\circ\text{C}$  to  $40^\circ\text{C}$ .

1 38. A process as claimed in claim 37, wherein the proteic substance is  
2 bovine serum albumin, human serum albumin or a gamma-globulin IgG, and  
3 the copolymer is Poly-(D-Glu,D-Lys) with a molecular weight of 20,000  
4 to 100,000 Daltons.

1 39. A process as claimed in claim 38, wherein the copolymer is poly-  
2 (D-Glu,D-Lys) having a molecular weight of 50,000 and a Glu:Lys = 6:4,  
3 the oxidized proteic substance copolymer molar ratio is equal to 1:15,  
4 the carbodiimide is used in excess from 3 to 6 mmol/1-10  $\mu\text{mol}$  of  
5 oxidized protein/poly-amino acidic copolymer; the carbodiimide is N-

6 ethyl-N'-(3-dimethylaminopropyl)-carbodiimide.

1 40. A process as claimed in claim 39, wherein:

2 - the oxidized protein/amino acidic copolymer conjugate is purified by  
3 dialysis on membranes with molecular exclusion limit cutoff ranging  
4 from 200,000 to 300,000, against a buffer at a pH of 5.0-6.0, at 0°C  
5 to 10°C;

6 - the hapten/carrier conjugate is purified by dialysis on membranes  
7 with molecular cutoff value ranging from 10,000 to 30,000 Daltons,  
8 against a buffer at a pH of about 7.0 to 7.5, at 0°C to 10°C.

1 41. A process as claimed in claim 40, wherein the hapten is selected  
2 among amino glycosidic antibiotics, amphotericine, cycloserine,  
3 amphetamine, sulphonylamides, cimaterol, and clenbuterol.

1 42. Process for the preparation of a conjugate of a hapten with a  
2 proteic substance, including the treatment of a hapten containing -C-  
3 OH groups with an oxidizing agent of the -C-OH groups to C=O groups,  
4 followed by treatment with the proteic substance and then with a  
5 reducing agent.

1 43. The process as claimed in claim 42, wherein the oxidation is  
2 carried out with  $\text{NaIO}_4$ , in water, at a pH ranging from 5.0 to 7.5, at  
3 a temperature ranging from 4°C to 40°C, shielded from light; excess  
4 oxidizing agent is eliminated by addition of glycerol or ethylene  
5 glycole; the reaction of oxidized hapten and the protein is typically  
6 carried out at an alkaline pH, at the temperature specified above for  
7 the oxidation.

1 44. A process as claimed in claim 43, wherein the hapten is selected  
2 among an amino glycosidic antibiotic, amphotericin, and idoxuridine.

1 45. Process for the preparation of a conjugate of a hapten containing

2 -NH<sub>2</sub> groups with a proteic substance, including the oxidation of the  
3 proteic substance with an oxidizing agent of the -C-OH groups to C=O  
4 groups, followed by treatment of the resulting oxidized proteic  
5 substance with the hapten and then with reducing agent.

1 46. A process as claimed in claim 45, wherein the oxidation is carried  
2 out with an inorganic periodate, in water, at a pH ranging from 5.0 to  
3 7.5, at a temperature ranging from 4°C to 40°C, shielded from light;  
4 the oxidizing agent is in stoichiometric excess with respect to the -  
5 C-OH of the proteic substance and, once the reaction is completed, its  
6 excess is eliminated by addition of a molar excess alcohol, at the  
7 oxidation temperature; treatment of the oxidized proteic substance  
8 with the hapten is carried out at an alkaline pH, at a temperature  
9 ranging from 4°C to 40°C.

1 47. A process as claimed in claim 45, wherein the hapten is selected  
2 among of neomycin, gentamicin, streptomycin and amphotericin.

1 48. Immunogen derivative as claimed in any of claims from 6 to 11.

1 49. Immunogen derivative obtainable according to the process as  
2 claimed in claims from 35 to 46.

1 50. Use of an immunogen derivative as claimed in any of claims 48 or  
2 49 in the preparation of IgGs active as specific antibodies to said  
3 drugs or metabolites.

1 51. Conjugate between by drug and/or metabolite and an enzyme which  
2 may be obtained according to the processes as claimed in any of claims  
3 36 to 47, wherein the proteic substance is an enzyme and the hapten is  
4 the drug or metabolite to be proportioned.

1 52. Use of a conjugate as claimed in any of claims 33 to 35 or 51, as  
2 a diagnostic reagent of the competitive-type ELISA assay.

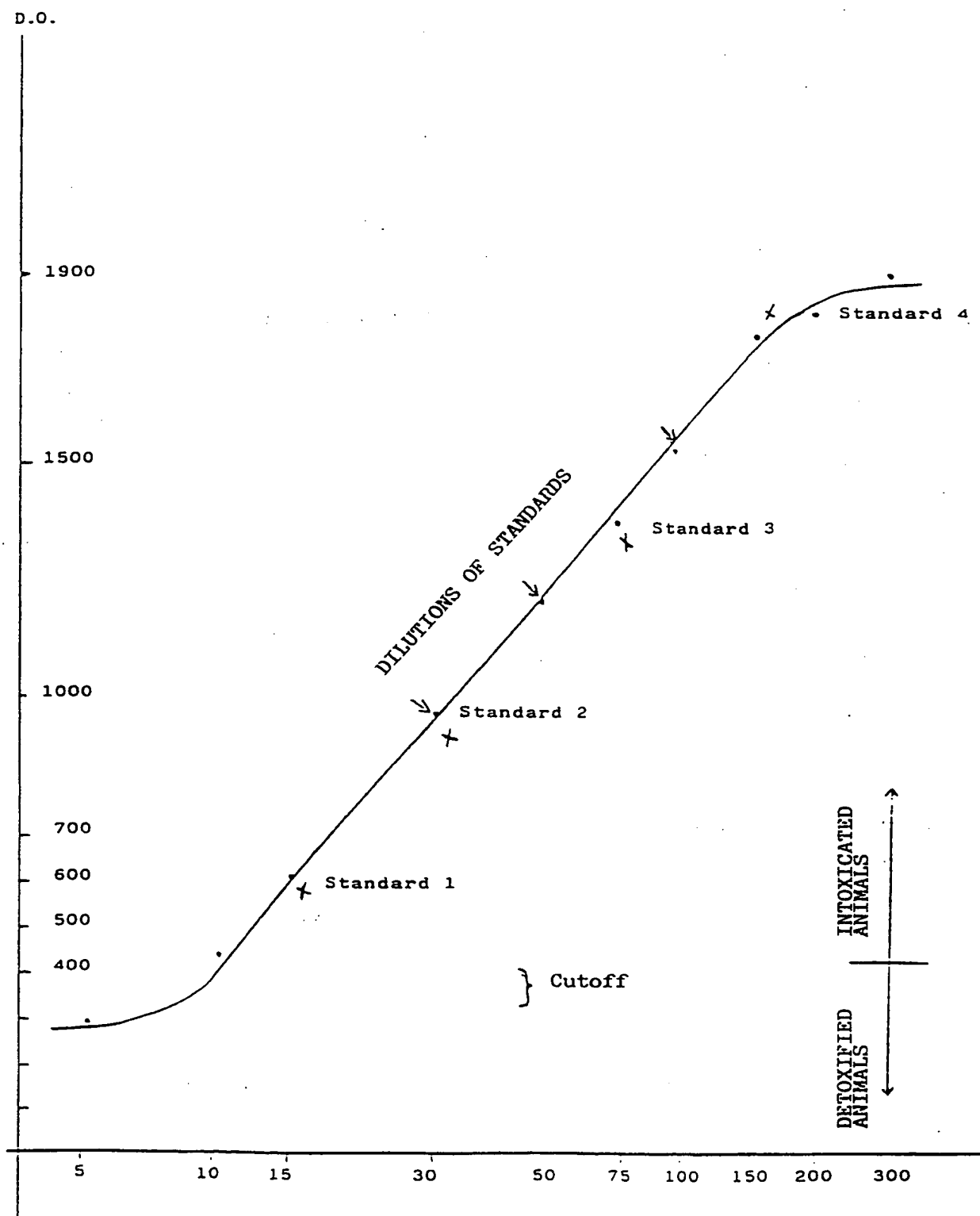
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1 53. A therapeutic method for detoxifying from a drug or a metabolite  
2 thereof a subject in a need of such treatment, comprising  
3 administering to said subject a therapeutically effective amount of a  
4  $F(ab')_2$  fragment as defined in claims 1 to 3 or 16, or a IgG  
5 immunoglobulin as defined in claims 24 or 25.

1 54. The therapeutic method as claimed in claim 53, wherein  $F(ab')_2$   
2 fragments are administered at a dose correspondign to  $F(ab')_2$ : drug or  
3 metabolite molar ratios from 1:1 to 1:5, and IgG are administered at a  
4 dose of 1 mole per at least 10 moles of drug present in circulating  
5 blood.



FIGURE 1



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# INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/EP 97/03358

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K16/44 C12P21/00 A61K39/395 G01N33/94 G01N33/543  
C12N9/96 A61K39/385 //C07K16/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	K. AOKI ET AL.: "Immunoassay for methamphetamine with a new antibody." FORENSIC SCIENCE INTERNATIONAL, vol. 44, no. 2-3, February 1990, IRELAND, pages 245-255, XP000673787 see abstract see material and methods	25, 32-35, 48-51
Y	---	1-12, 15-24, 26-28
Y	FR 2 301 266 A (COVAL) 17 September 1976  see the whole document ---	1-12, 15-24, 26-28
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### ° Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

18 November 1997

Date of mailing of the international search report

03.12.97

Name and mailing address of the ISA

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Authorized officer

Nooij, F

# INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/EP 97/03358

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>B. ROSE ET AL.: "Competitive indirect ELISA for ceftiofur sodium and the effect of different immunizing and coating antigen conjugates."  BIOCONJUGATE CHEMISTRY,  vol. 6, no. 5, September 1995, WASHINGTON, DC, USA,  pages 529-535, XP002031389  see the whole document</p>	<p>25,  29-31,  48-50</p>
X	<p>M. HURSTING ET AL.: "Tricyclic antidepressant-specific Fab fragments alter the distribution and elimination of desipramine in the rabbit: A model for overdose treatment."  JOURNAL OF TOXICOLOGY. CLINICAL TOXICOLOGY,  vol. 27, no. 1-2, 1989, NEW YORK, NY, USA,  pages 53-66, XP000673781  see the whole document</p>	<p>1,4,5,  16-20,  23-25,  27,29</p>
X	<p>D. MONGKOLSIRICHAIKUL ET AL.:  "Development of a latex agglutination inhibition reaction test for amphetamines in urine."  JOURNAL OF IMMUNOLOGICAL METHODS,  vol. 157, no. 1-2, 4 January 1993,  AMSTERDAM, NL,  pages 189-195, XP002031390  see abstract  * material and methods *</p>	<p>25,48-50</p>

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 97/03358

## **B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## **Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 97/03358

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 53 and 54 re directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No

PCT/EP 97/03358

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR 2301266 A	17-09-76	CA 1064396 A	16-10-79
		DE 2606118 A	26-08-76
		US 4093606 A	06-06-78
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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/03358

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FR 2301266 A	17-09-76	CA 1064396 A	16-10-79
		DE 2606118 A	26-08-76
		US 4093606 A	06-06-78
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